IN VITRO INDUCTION OF TETRAPLOIDY
IN DIPLOID BANANAS

Thesis submitted in part fulfilment of the requirements for the award of the
degree of **Doctor of Philosophy** in **Horticulture** to the
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Coimbatore - 641 003.

By

M.GANGA
(I.D. No: 98 - 815 - 003)

HORTICULTURAL COLLEGE AND RESEARCH INSTITUTE

TAMIL NADU AGRICULTURAL UNIVERSITY

COIMBATORE - 641 003

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CERTIFICATE

This is to certify that the thesis entitled **'*IN VITRO* INDUCTION OF TETRAPLOIDY IN DIPLOID BANANAS'** submitted in part fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN HORTICULTURE** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Ms. M. GANGA** under my supervision and guidance and that no part of this thesis has been submitted for the award of any degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

Place : Coimbatore
Date : 27.06.2001

Dr. N. CHEZHIYAN
Chairman

Approved by the advisory committee:

Chairman : **Dr. N. CHEZHIYAN**

Members : **Dr. N. KUMAR**

Dr. E. VADIVEL
(Co-opted Member)

Dr. P. BALASUBRAMANIAN

T. S. Raveendran
8-10:
Dr. T.S. RAVEENDRAN

Atul Chandra
External Examiner 8-10-2001
Date :

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M. GANGA SIVAKUMAR

ABSTRACT

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M. GANGA

Degree : **DOCTOR OF PHILOSOPHY IN HORTICULTURE**

Chairman : **Dr. N. CHEZHIYAN**
Professor and Head
Department of Spices and Plantation Crops
Horticultural College and Research Institute
Tamil Nadu Agricultural University
Coimbatore – 641 003.

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The present investigations on **'*In vitro* induction of tetraploidy in diploid bananas'** were undertaken with the objective of synthesizing banana tetraploids by inducing chromosome doubling in diploid clones employing anti-mitotic agents. The diploid clones involved were Sannachenkadali (AA), Anaikomban (AA), Kunnan (AB) and Thattillakunnan (AB). The cv. Sannachenkadali was chosen for its resistance to leaf spot disease (*Mycosphaerella* spp.), Anaikomban for its resistance to burrowing nematode (*Radopholus similis*), Kunnan for its tolerance to leaf spot, burrowing nematode and Panama disease (*Fusarium oxysporum* f.sp. *cubense*), and Thattillakunnan for its tolerance to burrowing nematode/ The anti-mitotic agents employed were colchicine and oryzalin.

Shoot tips of the diploid clones were subjected to treatment with either of the anti-mitotic agents, colchicine and oryzalin and cultured *in vitro* on Murashige and Skoog (1962) medium. Both the anti-mitotic agents had a tendency to suppress the *in vitro* regeneration, the degree of suppression caused being higher with colchicine than with oryzalin. A peculiarity observed in the *in vitro* regeneration phase was a growth stimulation effect of oryzalin which resulted in the number of multiple shoots regenerated from the oryzalin-treated cultures surpassing the untreated control. The degree of decline in the regeneration rates had a positive relationship with the concentration of the anti-mitotic agent as well as the duration of treatment. The negative effect associated with prolonged treatment duration was higher with colchicine than with oryzalin. The cultivars varied in their response to the two anti-mitotic agents.

Regenerants derived from colchicine- and oryzalin-treated cultures were subjected to preliminary ploidy screening based on stomata and chloroplast analyses, followed by confirmation of ploidy status based on chromosome counting. Plants with stomatal densities ranging between 12.00 and 19.00 stomata mm^{-2} and stomatal dimensions (length x breadth) between 1850 and 2250μ^2 were categorized as tetraploids. Those with stomatal densities of 39.00-50.00 stomata mm^{-2} and stomatal dimensions of 950-1200μ^2 were categorized as diploids, while those with densities below 8.00 stomata mm^{-2} and dimensions above 2500μ^2 were classified as octoploids. Those with mixtures of two or more of these stomatal densities and dimensions were categorized as mixoploids. The octoploids and mixoploids were collectively categorized as 'other' ploids. The average stomatal size of the tetraploids was 1.91 times as great as that of the corresponding diploids.

It was observed that the chloroplast density per guard cell pair of the stomata was higher for the induced tetraploids. The densities fell within a definite range for the diploids and tetraploids (9.00-11.00 and 15.00-17.00 chloroplasts per guard cell pair respectively), enabling categorization of plants into different ploidy classes. Doubling of the ploidy level from 2x to 4x increased the chloroplast density by a factor of approximately 1.70. Ploidy verification based on stomatal traits and chloroplast traits proved to be 82.19 % and 95.89 % reliable, respectively.

Many bottlenecks were encountered with during the process of counting the somatic chromosomes of root tip cells. The very low frequencies of dividing cells, non-selective staining of tissues, difficulty in identification of chromosomes due to their very small size and poor staining of cells due to the rigid cell wall characteristic of *Musa* sp. were some of them. Owing to such problems, the chromosome numbers in cases where exact counting could not be done were corrected to the nearest possible euploid number, following earlier reports on identical works (White, 1928; Cheesman, 1932a,b). During such cases of uncertainty, chloroplast counts were also taken as a supporting evidence, since they have proved to be highly reliable.

The rates of tetraploidy induced increased with increase in the concentration of the anti-mitotic agent. Among the colchicine treatments, the highest rate of tetraploidy (17.09 %) was induced by the one which involved exposure of cultures to 7.5mM colchicine for 12h. Among the oryzalin treatments, the highest rate of tetraploidy (26.53 %) was induced by the treatment involving 40μM oryzalin for 3d. Thus it was observed that oryzalin at a concentration 187.5 times lower than that of colchicine had a higher chromosome doubling efficiency. Based on the higher chromosome doubling efficiency and lower phyto-toxcity (assessed based on symptoms associated with toxicity observed in the *in vitro* cultures and rates of 'other' ploids induced), oryzalin proved to be superior to colchicine.

The four diploid cultivars showed differential response to the anti-mitotic agents. The cultivars with genomic constitution 'AA' namely, Sannachenkadali and Anaikomban produced comparatively higher rates of tetraploidy (15.31% and 16.11%, respectively with colchicine treatments and 20.19% and 17.19%, respectively with oryzalin treatments), than the cultivars with the genomic constitution 'AB', namely Kunnan and Thattillakunnan.

The induced tetraploids had slower growth rates during the initial days (at transfer and up to two months after transfer to *ex vitro* environment). The pseudostem height, girth and crop growth rates which were initially lower than those of the corresponding diploids, surpassed them at later stages (at four months after transfer and afterwards) resulting in morphological gigantism. The chlorophyll contents and photosynthetic efficiencies of the induced tetraploids were higher. The red-pigmented cv. Sannachenkadali had higher anthocyanin contents than the corresponding diploid plants. The interphase nuclear volume of the tetraploids was higher than their corresponding diploids. The induced tetraploids had leaves with lower mechanical strength which was reflected by their lower petiole dry weight percentages.

The induced tetraploids are now in their vegetative phase. Further evaluation of these plants to assess their genomic constitution, fertility status, disease resistance, economic traits, crossability with diploids to produce triploids etc., will be taken up in the future and the potential tetraploids would be identified and used in future breeding programmes. They may be crossed with the potential synthetic diploids which have been developed at the Tamil Nadu Agricultural University (Sathiamoorthy, 1987) to produce improved secondary triploids.

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CHAPTER I

INTRODUCTION

The national economies of many of the developing countries depend largely on dessert bananas as an export crop. According to FAO, more than 58 million tonnes of bananas and 30 million tonnes of plantains were produced worldwide in the year 2000. India is the largest producer of bananas (11 million tonnes) and Uganda the largest producer of plantains (9 million tonnes). Nearly half of the world's bananas are grown in Asia, while almost 75 per cent of the world's plantains are grown in Africa. Bananas and plantains are staple food crops for millions of people in developing countries. In terms of gross value of production, bananas and plantains are the developing world's fourth most important crop after rice, wheat and maize.

Dessert bananas are mainly triploids and they are increasingly threatened by *Fusarium* wilt (*Fusarium oxysporum* f.sp. *cubense*) and black Sigatoka (*Mycosphaerella fijiensis* var. *difformis*) diseases. The foremost aim in banana and plantain improvement programmes, besides improving the yield, is to synthesize new varieties with increased resistance or tolerance to the major diseases (Persley and de Langhe, 1987). However, hybridization of most commercially acceptable bananas and plantains is nevertheless complicated due to their parthenocarpic nature and triploidy, resulting hardly in any seed set. This necessitates generation of newer triploids with disease resistance and other favourable attributes.

Tetraploidy being the major evolutionary pathway between diploidy and triploidy in *Musa*, could well be used for the purpose of breeding triploids (Stover and Buddenhagen, 1986). This has necessitated new approaches to polyploidy breeding including attempts at *in vitro* manipulation of the genetic potential of *Musa*. Further, production of secondary triploids by crossing primary triploids with improved diploids being less efficient, the alternative procedure involving induction of chromosome doubling of promising diploids followed by crossing them with diploids has been identified as a reliable technique (van Duren *et al.*, 1996). Production of triploid hybrids by crossing autotetraploids with improved, disease resistant diploids has also been identified as a promising way to increase productivity (Hamill *et al.*, 1992).

The scarcity of natural and disease resistant tetraploids makes it imperative that new tetraploids are being synthesized. In this regard, *in vitro* techniques have been initiated in banana elsewhere, but not in India. A breeding scheme involving mass production of tetraploids from improved diploids through seed treatment with colchicine was proposed as long as 1967 (Vakili, 1967). However, until recently, very little work has been done on the production of autotetraploids from vegetative material. Hamill *et al.* (1992) and van Duren *et al.* (1996) recovered autotetraploids from the *Musa acuminata* clone SH-3362 which has a high level of resistance to black Sigatoka and race 4 of *Fusarium* wilt.

Polyploidy has featured prominently in natural plant evolution enabling the establishment of populations with wider adaptability and superior potential than their parents (Stebbins, 1950, 1971; de Wet, 1980). Polyploidy was used initially out of curiosity because of increased vigour and for restoring fertility in sterile hybrids. However, research in the 1980's has further elaborated the role polyploidy can play in regulating hybrid fertility and genetic introgression between species.

The discovery of the use of colchicine for chromosome doubling in the 1940's and production of haploid plants from anther culture in the 1960's raised great hopes for the use of ploidy manipulations. Polyploidy has significant roles to play:

1) in overcoming cross-incompatability, by regulating ploidy level in gametes, endosperm or embryo.
2) in restoring fertility in sterile species hybrids.
3) in serving as a genetic bridge to transfer genes between species.

Induced autopolyploidy may play an important role in enhancing crossability and in facilitating gene flow (Singh *et al.*, 1990). Thus induction of tetraploidy in the banana crop could have a significant impact on breeding programmes aimed at transferring valuable traits present in diploid accessions and not easily accessible due to the genomic nature of the cultivated banana cultivars.

With the above facts in view, the present investigations on **"*In vitro* induction of tetraploidy in diploid bananas"** were undertaken at the Tissue Culture Laboratory of the Department of Fruit Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, with the following objectives:

1. To standardise a method for inducing tetraploidy in diploid starting material under *in vitro* conditions.
2. To standardise early screening methods for verification of ploidy status of the *in vitro* derived plantlets.
3. Evaluation of the *in vitro* induced tetraploids.

The cultivars Sannachenkadali (AA), Anaikomban (AA), Kunnan (AB) and Thattillakunnan (AB) were the diploid starting materials involved in the investigations. The cv. Sannachenkadali was chosen for its resistance to leaf spot disease (*Mycosphaerella* spp.), Anaikomban for its resistance to burrowing nematode (*Radopholus similis*), Kunnan for its tolerance to leaf spot, burrowing nematode and Panama disease (*Fusarium oxysporum* f.sp. *cubense*) and Thattillakunnan for its tolerance to burrowing nematode. The anti-mitotic agents employed to induce tetraploidy through chromosome doubling were colchicine and oryzalin.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1. History of polyploidy breeding in banana

Less than one-half of the banana clones are diploid and almost all the remainder are triploid (Stover and Buddenhagen, 1986). The diploids are utilized in some local production, but on a relatively small area/scale. Triploids have been much more successful than diploids because of their greater productivity, vigour, sterility and range of useful variability. During the past 70-75 years, one of the targets in banana breeding has been to obtain a useful tetraploid, from an initial addition to an unchanged triploid Gros Michel or a shorter version of it, of an "n" genome from a diploid. This is because, tetraploidy is considered to be the major evolutionary pathway between diploidy and triploidy in *Musa* and hence tetraploidy could well be used for the purpose of breeding improved triploid varieties.

Banana breeding began in 1922 at the Imperial College of Tropical Agriculture in Trinidad and in 1924 in Jamaica. A breeding programme was initiated in Honduras in 1960. The objetive of all these programmes was only to obtain, at first, a fusarial wilt resistant Gros Michel, and later, resistance also to Sigatoka leaf spot in a dessert banana for the export trades, to compete with the fusarial wilt resistant Cavendish group. At first, wild diploids of *M. acuminata* ssp. *malaccensis* were used as males. To improve the agronomic characteristics of the male, diploid breeding programmes were initiated in Trinidad, Jamaica and Honduras that resulted in greatly improved bunches. These diploids (2n = 22), crossed onto Highgate (a shorter mutant of Gros Michel), produced a majority of tetraploids (2n = 44) and a few heptaploids. However, these tetraploids had the following defects:

- Agronomical inferiority of the tetraploid karyotype due to a greater contribution from Highgate.
- Low level of fertility (an average of only 1 –3 seeds per pollinated bunch)
- The possibility of producing an occasional hard seed in the fruit since tetraploids undergo normal meiosis and produce abundant pollen.

Simmonds (1966) observed that when primary tetraploids were crossed with the improved diploids, secondary triploids were produced. These were long considered to be useless, and the triploid approach to improving bananas received almost no attention until about 1980. During the eighties, triploid breeding schemes began to be given a reconsideration taking into view the need for newer triploids as the dessert bananas available then were being increasingly threatened by many dreaded diseases. It was here that the need for synthesizing new disease resistant tetraploids was strongly felt, since natural tetraploids were scarce and these scarcely available natural tetraploids were associated with one problem or the other.

2.1.1. Natural tetraploids of banana

Richardson *et al.* (1965) stated that although uncommon, it is not unlikely that many natural tetraploids exist. Prior to 1965 only one natural tetraploid was considered to exist, viz., Klue Teparod, which was also later proved of its non-tetraploid state. When 648 accessions of the United Fruit Company of Honduras were examined, only six natural tetraploids were found (Richardson *et al.*, 1965). In Papua New Guinea, 13 accessions among 200 were listed as tetraploids (Shepherd *et al.*, 1987). Only one natural pure *acuminata* tetraploid is known to exist and this is from Papua New Guinea. Thus, the few natural tetraploids existing are confined to South East Asia and are mostly from primitive agricultural areas.

Naturally occurring tetraploids

Group Name	Genome	Distribution	Reference
- Atan	AAAB	New Britain	Richardson *et al.* (1965)
- Kalamazol	AABB	Solomon Islands	Richardson *et al.* (1965)
- Papua New Guinea accessions No. of accessions :			Shepherd *et al.* (1987)
11	AAAB	Papua New Guinea	
	AABB		
1	ABBB	Papua New Guinea	
1	AAAA	Papua New Guinea	

Since most tetraploids were found in areas where edible cultivars were grown haphazardly in mixture under very primitive agriculture, Vakili (1967) suggested that tetraploidy was one of the evolutionary pathways between diploidy and triploidy.

2.1.2. Synthetic tetraploids of banana:

Cheesman and Dodds (1942) reported on a mechanism for the production of tetraploids by intensive pollination of triploids with haploid pollen. Tetraploids were produced by crossing female AAA, AAB and ABB with AA and BB diploid males. Synthetic tetraploids namely, IC2 (AAAA) from Gros Michel x *M. acuminata* ssp. *malaccensis* , Bodles Altafort (AAAA) from Gros Michel x Pisang Lilin and a tetraploid of the genomic constitution ABBB from Neyvannan x *M. balbisiana* clone Sawai were bred in Trinidad, Jamaica and India respectively (Simmonds, 1962; Bakthavatsalu *et al.*, 1970). Some normal tetraploid progenies with high level of resistance to Sigatoka disease were produced by pollinating the fertile triploid plantain Laknau (AAB) with diploids (AA). One such tetraploid is 64-2596 (Rowe, 1976).

Swennen and Vuylsteke (1988) investigated the potential of non-conventional breeding techniques that would complement and support conventional activities. They reported that secondary triploids could be produced at a very low efficiency by crossing primary triploids with improved diploids. An alternate procedure to obtain synthetic triploids by inducing chromosome doubling of promising diploids and then crossing them with diploids was reported by Novak (1992). Hamill *et al.* (1992) viewed the production of triploid hybrids by crossing autotetraploids with improved, disease resistant diploids as a promising way to increase productivity.

Vakili (1967) proposed a colchicine-induced tetraploid breeding scheme for *M. balbisiana* and *M. acuminata* (ssp. *banksii, errans, microcarpa*). Hamill *et al.* (1992) and Smith *et al.* (1993) recovered autotetraploids from the diploid clone SH-3362 by treating shoot tips with colchicine. van Duren *et al.* (1996) recovered high frequencies of non-chimeric tetraploids from colchicine and oryzalin treated *in vitro* cultures of the *M. acuminata* clone SH-3362. Asif *et al.* (2000) induced autopolyploids of the wild banana *M. acuminata* ssp. *malaccensis* by colchicine treatment of *in vitro* cultured zygotic embryo-derived shoot apices.

2.2. Significance of polyploids

2.2.1. Functional significance of polyploidy

The occurrence and physiological significance of somatic cell polyploidy have been reported by several authors (Guttler, 1953; Brodsky and Uryvaeva, 1977; Nagl, 1978; D'Amato, 1952, 1977, 1984) according to whom independently of its mode of origin, polyploidy being a multiplication of the genome, the increased availability of nuclear templates was favourably reflected in the growth of the protoplast and its functional activity. According to Brodsky and Uryvaeva (1977), Nagl (1973, 1978) and Clutter *et al.*(1974), in plant and animal cells, the amount of RNA synthesized was related to the ploidy level and in polyploid cells, the transcriptional activity per unit amount of DNA was twice that in the diploid cells. Further, they observed a positive correlation existing between the number of plastids in a cell and the nuclear DNA amount in differentiating cells, the duplication of the DNA amount entailing an increase in plastid number of about 70%.

2.2.2. Sources of disease resistance

Tetraploid clones of banana developed in Jamaica and Honduras have been found to have high resistance to races 1 and 2 of fusarial wilt and moderate resistance to leaf spot. However, the first tetraploid clone released (IC2) produced in Jamaica around 1930, succumbed to fusarial wilt when grown in Honduras. The second clone (Bodles Altafort) was reported to be attacked by race 2 of fusarial wilt in India (Stover & Simmonds, 1987). Two promising tetraploids from Honduras resulting from a cross between Cocos (a semi-dwarf mutant of Gros Michel) with Pisang Lilin on the diploid side were resistant to fusarial wilt. However, they proved susceptible to race 4, when tested in Taiwan (Hwang and Ko, 1980). The fertile triploid plantain Laknau (AAB) when pollinated with diploids (AA), produced some normal tetraploid progenies (Rowe, 1976). One such tetraploid 64-2596, though a low yielder, showed complete resistance to Sigatoka disease.

2.2.3. Improved characteristics of banana tetraploids

Richardson *et al.*(1965) opined that due to weak agronomic characters which were a natural tendency of tetraploids, they were less likely to have been selected for edible types by local people in primitive agriculture. However, there are reports on tetraploids with improved characteristics . A breeding scheme at IITA aimed at producing black sigatoka-resistant tetraploid hybrids from 3n x 2n crosses involving 37 cultivars of the plantain subgroup (AAB) as female parents and the diploid male fertile clone Calcutta 4 a wild seeded banana belonging to the *Musa acuminata* ssp. *burmannicoides* and 'Pisang Lilin', an edible diploid related to *M.acuminata* ssp.*malaccensis* (both resistant to black sigatoka disease), resulted in 18% tetraploids. These tetraploids were associated with the following improved characteristics:

a) reduced severity of black sigatoka disease
b) higher bunch weight
c) occurrence of fruit parthenocarpy
d) shorter plant stature
e) better ratooning
f) absence of the tendency to leaf petiole breakage and finger drop (which are possible defects of tetraploids).

The only defect observed in these tetraploids was longer fruit filling times compared to their plantain parents (Vuylsteke *et al.*, 1993).

2.2.4. Breeding potential

Shepherd (1987) reviewed the results of tetraploid breeding and opined that in all the cases, the tetraploid hybrids showed the expected resemblance to the female parent, which constituted three quarters of the genotypes. This led him to conclude that tetraploids offered the possibility of adding specific characters from a selected diploid male parent to a cultivar. The low potency of pollen produced by tetraploids was considered to act as an effective barrier against self fertilization.

2.3. Types of polyploidy

In agreement with D'Amato (1977, 1984), Pera (1970) and Brodsky and Uryvaeva (1977), the term 'cell polyploidy' is used to define a multiple (2n) doubling of nuclear DNA independently of mechanisms responsible for the doubling. According to D'Amato (1989), the types of cell polyploidization in plants are summarized as follows.

2.3.1. Acytokinetic mitosis

The absence of cytokinesis at the end of mitosis results in a binucleate cell. If the two nuclei undergo one or more acytokinetic mitoses, a multinucleate cell is formed. This type of polyploidization occurs in some angiosperms when the protoplasts are released from the tapetal cells into the anther loculus.

2.3.2. Spindle fusion

Since the two nuclei of a binucleate cell generally divide synchronously, there is opportunity especially in smaller cells, for spindle fusion, viz., the fusion of metaphase plates and/or anaphase poles. Spindle fusion is a characteristic method of polyploidization in some plant systems: e.g., multinucleate tapetal cells.

2.3.3. Restitutional mitosis

Restitutional mitosis results from spindle disturbances at anaphase or rarely, at metaphase, leading to the production of polyploid restitution nuclei. Spindle disturbances may frequently be associated with defective movement of individual chromosome (laggards) and / or with chromosome stickiness which favours sticky bridges at anaphase and chromosome clumping at metaphase. Restitutional mitosis is not rare in such short-lived plant tissues as the anther tapetum and the endosperm.

2.3.4. Endomitosis

In endomitosis, the chromosomes enter a prophase stage (endoprophase), then they contract further (endometaphase), their chromatids separate parallel to each other (endoanaphase) and finally decondense to take the resting nuclear structure. The endomitosis – a process that produces a doubling of the endomitotic cycle essentially differs from mitosis for the absence of mitotic spindle, and so, it has been likened to a colchicine-arrested mitosis, apart from the much lower degree of chromosome contraction in endomitosis. Thus the term 'endomitosis' must be reserved to those cases of chromosome cycle comparable to that of mitosis occurs inside the nuclear envelope.

2.3.5. Chromosome endoreduplication

Chromosome endoreduplication is an endonuclear chromosome duplication that occurs in interphase in the absence of any obvious condensation and decondensation stages, excluding the dispersion of chromocentres. Endoreduplication is the commmmonest and most widespread method of somatic polyploidization in plant cells.

2.4. Methods of polyploidization in *Musa* spp.

2.4.1. 3n x 2n method

Cheesman and Dodds (1942) reported on a mechanism for the production of tetraploids (Fig.1a). They observed that under experimental conditions, intensive pollination of triploids with haploid pollen yielded - when any seed was produced - a majority of tetraploids, a few triploids and a small minority of heptaploids and diploids. Tetraploids resulted from crosses between female AAA (Ambey and Wasolay), AAB (Tomnam and Laknau) with AA diploid males and between female ABB (Awak, Bluggoe, Champa Madras, Ice Cream, Tip) with AA and BB diploid males.

Synthetic tetraploids namely, IC2 (AAAA) from Gros Michel X *M. acuminata* ssp. *malaccensis* , Bodles Altafort (AAAA) from Gros Michel X Pisang lilin and a tetraploid (ABBB) from Neyvannan x *M.*

balbisiana clone Sawai bred in Trinidad, Jamaica and India respectively (Simmonds, 1962; Bakthavatsalu *et al.* 1970) and the tetraploid 64-2596 (Rowe, 1976) produced by pollinating the fertile triploid plantain Laknau (AAB) with diploid (AA) were synthesized by the 3n x2n method.

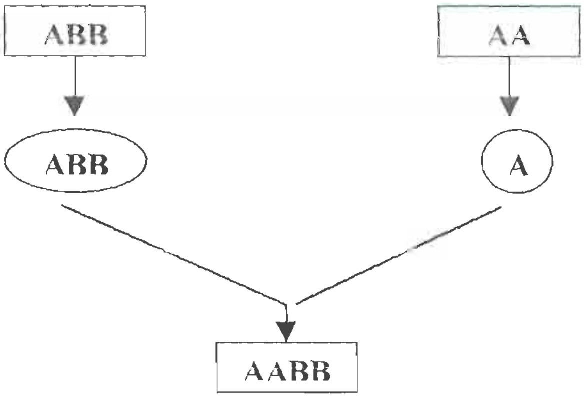
2.4.2. <u>4n x 3n method</u>:Crosses undergoing uneven meiosis (Fig.1b)

2.4.3. <u>4n x 4n method</u>:Crosses undergoing single restitution Fig.1c)

These two methods have also been reported (Simmonds and Shepherd, 1955) as possible routes of formation of tetraploids.

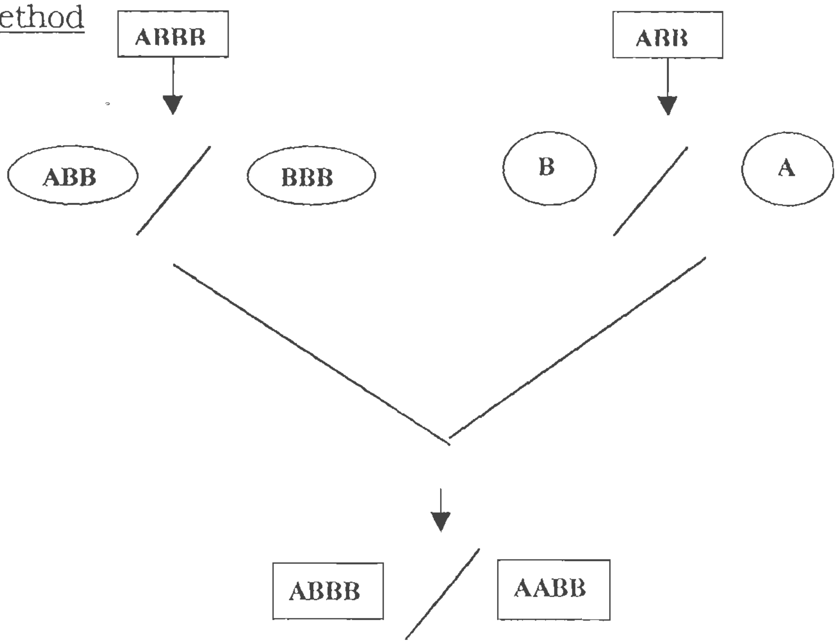
Fig. 1. Routes of formation of synthetic tetraploids

1a. <u>3n x 2n method</u>



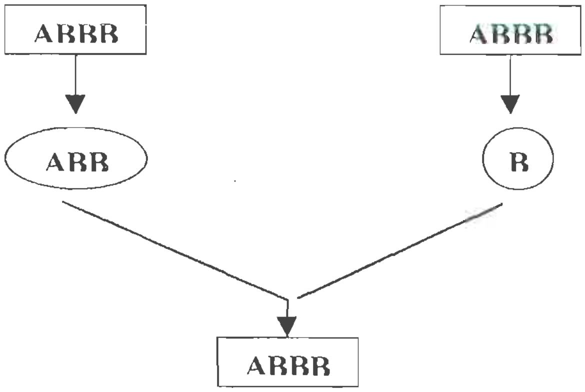
Single restitution

1b. <u>4n x3n method</u>



Uneven meiosis

1c. 4n x 4n method



Single restitution

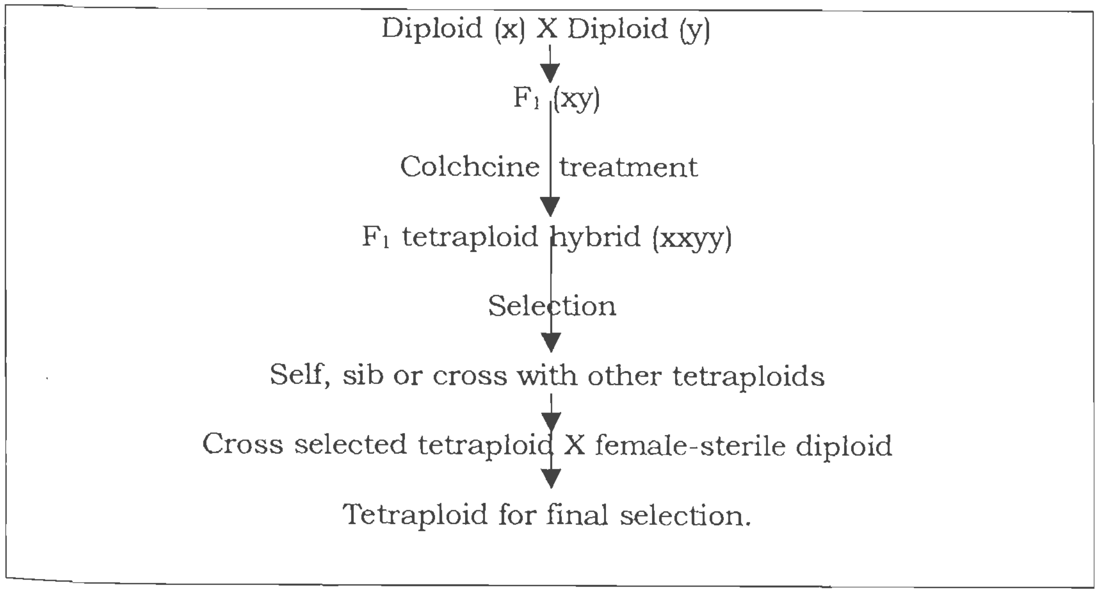
2.4.4. Inducing polyploidy using anti-mitotic agents

There are reports on polyploidy induction in *Musa* spp. under *in vivo* and *in vitro* conditions.

2.4.4.1. *In vivo* polyploidy induction

Vakili (1967) found that tetraploidy could be readily induced with colchicine in *M.balbisiana* and *M.acuminata* (ssp. *banksii, errans* and *microcarpa*). He proposed a colchicine-induced tetraploid breeding scheme for producing tetraploids. Polyploidy was induced by means of immersing newly germinated seedlings in 0.5% colchicine solution by the following method.

Fig. 2. *In vivo* polyploidy induction scheme proposed by Vakili (1967)



Stover and Buddenhagen (1986) suggested that, in contrast to the 3n x 2n method which produced very few tetraploid hybrids, the method proposed by Vakili (1967) could mass produce tetraploids from various improved diploids. These could be screened rigorously for disease resistance and all other characters and even undergo repeated crossing and selection cycles, as in diploids. The best could be crossed with superior diploids to yield triploids in large numbers. By having numbers of triploids from diverge crosses available, and with rigorous disease screening at the seedling stage, the chances for obtaining a superior triploid clone would be greatly increased. The best triploids could even be reused (as the variety 'Highgate' is used), for obtaining a new tetraploid in a recurrent breeding system to initiate new tetraploid x diploid cycles with a triploid target.

Vuylsteke *et al.* (1993) emphasized that promising black Sigatoka-resistant tetraploid hybrids of plantain which combined high yields, adequate plant height and improved ratooning could be produced by using a wild diploid banana as male source of disease resistance.

2.4.4.2. *In vitro* polyploidy induction

Hamill *et.al.*, (1992) induced fusarial wilt resistant autotetraploids from the diploid clone SH-3362 by treating the shoot tips wiith 0.5% colchicine for two hours. Although these polyploids were weak, produced few suckers and seemed to be even more cold resistant than the diploid clone SH-3362, they maintained their level of resistance to race 4 *Fusarium* wilt. Culturing shoot tips in liquid medium supplemented with 5.0 mM colchicine for 48 hours or 30μM oryzalin for seven days in combination with 2%(v/v) DMSO resulted in high frequencies (23.1% and 29.1%) of non-chimeric tetraploids in the fourth vegetative generation (van Duren *et al.*, 1996). Although mixoploidy persisted in subsequent cycles of vegetative propagation, tetraploids as identified by flow-cytometry remained solid non-chimeric during two more cycles.

Smith *et al.* (1993) devised a technique for the induction of tetraploid plants from a micro propagated diploid clone SH-3362. Colchicine was applied to actively growing shoot tips *in vitro* which resulted in 30% tetraploidy. The optimum concentration was 0.5% w/v colchicine with 2% w/v DMSO applied for 2 or 4h. Asif *et al.* (2000) induced autopolyploidy in the wild banana *M. acuminata* ssp. *malaccensis*. Use of zygotic embryo culture for large scale production of banana seed progenies and improved media compositions coupled with treating shoot apices with 0.5% colchicine in combination with 2% DMSO for 2 hours facilitated the process of polyploidy induction.

2.5. Anti-mitotic agents for polyploidy induction

There are certain chemicals or groups of chemicals, which are known to influence strikingly certain phases of cell divisions. These phases are

1) the entering of cells into division
2) the formation of a functioning spindle, and
3) cytokinesis.

Agents which prevent cells from entering mitosis inhibit the division of the cell, the nucleus and the chromosomes. However, though they inhibit the formation of free chromatids or daughter chromosomes, they do not necessarily inhibit chromosome reproduction. The stage affected is interphase, and sometimes early prophase, which then is caused to revert to the interphase condition. When these "preprophase inhibitors of mitosis" (D'Amato, 1949) act on later stages of prophase, they often inhibit the normal evolution of prophase to metaphase, an effect called "prophase poisoning" by D'Amato (1949, 1952).

2.5.1. Colchicine

Today, a large number of agents with ability to suppress spindle function are known. However, the first of these agents to be discovered, colchicine, is still the most commonly used. The drug colchicine was isolated for the first time from the bulbous plant *Colchicum autumnale.* Studies on the cytological effects of colchicine were stimulated by Ludford (1936) who was the first to recognize the colchicine effect as an inhibition of spindle function resulting in arrested mitosis and Blakeslee and Avery (1937) who discovered that colchicine was able to induce chromosome doubling in plants. Other cytological studies on the effect of colchicines in plant material are those by Gavaudan and Gavaudan (1937), Dustin *et al.* (1937), Eigsti (1938) and Nebel and Ruttle (1938).

The cytological and genetic significance of colchicines was first explained by Nebel and Ruttle (1938). To induce chromosomal changes in somatic tissues of cultivated plants as obtained by inhibition of spindle formation caused by colchicine is referred to as 'plant breeding with non-Mendelian methods' by the authors.

The chromosome-doubling ability of colchicine is attributed to its affinity for the spindle (Cornman, 1942). The colchicine-effect has been reported to be due to a specific impairment of spindle function which results in a blockage in the completion of division in actively dividing cells. Gaulden and Carlson (1951) observed that in addition to the spindle fibres serving as a substrate for colchicine, there existed an enzyme specificity between the drug and the spindle. Inhibition of spindle function leads to prevention of both cell division and nuclear division, while the chromosomes reproduce and divide to form separate daughter chromosomes. As a result, the number of chromosomes per cell will be doubled for each mitotic cycle in the presence of the inhibitor.

According to Levan (1938), the modification of mitotic behaviour induced by colchicine consisted of an inactivation of the spindle apparatus connected with a delay of the division of the centromere. The stage delayed by colchicine corresponded to the metaphase of the normal mitosis, and for this reason colchicine is said to cause an "accumulation of metaphases". Further, in colchicine-treated cells, the divisions did not always take place quite simultaneously within all the chromosomes in the cell, which indicated that they were desynchronized, as well as delayed (Levan, 1938).

The relationship between c-mitotic activity and chemical structure of colchicine and its derivatives was studied by Steinegger and Levan (1947, 1948), who found that the c-mitotic activity was very sensitive to modifications of the colchicine molecule. Colchicine is the methyl ether of colchiceine. Colchicine exists in two tautomeric forms, namely, colchicine and isocolchicine. Isocolchicine was about 100 times less effective than colchicine as an inducer of c-mitosis in *Allium cepa.*

When colchicine is applied to plant cells, cytoplasmic microtubules rapidly depolymerize. Colchicine binds to the major constituent protein of microtubules, tubulin. The tubulin-colchicine complexes inhibit microtubule polymerization by binding to the microtubule ends (Margolis and Wilson, 1977; Margolis *et al.*, 1980; Bergen and Borisy, 1983, 1986; Farrel and Wilson, 1984).

Colchicine was employed to induce polyploidy in Musa species by several authors. Vakili (1967), Hamill *et. al.* (1992), Smith *et.al.* (1993), van Duren *et. al.* (1996) and Asif *et. al.* (2000) induced polyploidy in *Musa* spp. using colchicine.

2.5.2. Oryzalin

The chromosome-doubling ability of oryzalin is also attributed to its affinity for the spindle (Upadhyaya and Nooden, 1977). Oryzalin being a dinitroaniline herbicide, has an affinity for tubulin, a microtubule-subunit protein. It binds to tubulin forming a highly stable oryzalin-tubulin complex leading to microtubule depolymerization. Oryzalin has been reported to cause spindle inhibiton in yet another way also. At particular concentrations, it disturbs the Ca^{2+} transport systems operating in cell organelles. This leads to microtubule depolymerisation since Ca^{2+} has an improtant role to play in the regulation of microtubule assembly (Weisenberg, 1972; Haga *et al.*, 1974; Olmsted and Borisy, 1975; Fuller *et al.*, 1975). Microtubule depolymerization leads to spindle disfunctioning, ultimately resulting in a doubled/higher chromosome number.

Oryzalin has been reported to induce polyploidy in a number of plant species (Morejohn and Fosket, 1984; Falconer and Seagull, 1987; Sree Ramulu et. al. ,1991 and Tosca et. al., 1995). Polyploidy was induced in the *M. acuminata* clone SH-3362 by culturing shoot tips in liquid medium supplemented with 30μm oryzalin (3,5-dinitro-N4, N- dipropylsulphate) for seven days (van Duren *et al.*, 1996).

According to Strachan and Hess (1983), plant responses to micromolar concentrations of dinitroaniline herbicides (DNH) were similar to those to millimolar concentrations of colchicine. They found oryzalin to have a higher microtubule depolymerizing efficiency compared to colchicine, and attributed this to the high specificity of binding of oryzalin to tubulin followed by the formation of a DNH-tubulin complex which had a stronger capacity than that of the colchicine-tubulin complex to prevent polymerization of tubulin into microtubules, leading to disfunctioning of the spindle fibres.

2,5.3. Other antimitotic agents

The herbicides benefin, amiprophos-methyl and trifluralin are involved in inducing polyploidy in plant species. These compounds have strong binding affinity to plant tubulins (Morejohn and Fosket,1984; Falconer and Seagull, 1987; Upadhyaya and Nooden; 1977, 1980; Falconer et. al., 1988).

2.6. Impact of polyploidy

2.6.1. Morphological characteristics

Wettstein (1927) demonstrated that the morphological alterations in experimental polyploids were ultimately due to alterations in cell size. As is well known, there is a positive correlation, though not strict proportionality, between chromosome number and cell volume. Muntzing (1935) summarized the morphological alterations in tetraploids as production of taller plants, thicker and stouter stems, larger and thicker leaves which are relatively shorter and broader and darker green, larger flowers, floral parts and seeds.

Richardson *et al.* (1965) observed that natural and bred tetraploids of banana were tall plants (3-5m) with drooping, weak petioles. Vakili (1967) observed that tetraploids of *M. balbisiana* in comparison with diploids produced fewer roots, had droopy, fragile leaves, produced fewer suckers that took longer to emerge, were slower ratooning and had smaller bunches but usually larger fruits. The most conspicuous feature of the tetraploids were their drooping leaf habit and doubling of the leaves at leaf bases during the later stages of their growth as described below.

Growth habit of colchicine-induced tetraploids of *M. balbisiana* compared with diploids (Vakili, 1967)

Character	Diploids	Tetraploids
Roots per 1000 cc rhizome	114	36
Pseudostem		
Height in m	4.3	4.9
Circumference at 1 m above ground (cm)	63	79
Leaf		
Habit	Erect	Drooping
No. emerged/plant/month	3.8	4.3
Suckers		
No. per plant	5.06	3.0
Emergence time (months)	5.9	8.4
Bunch		
Shooting time (months)	13	15.5
Average number of hands	10	8
Fruit		
Relative size	1	0.5-2

Vakili (1967) analysed the relationship between the ploidy and leaf pigmentation in *Musa*.spp. He observed that when the seedlings of certain clones of *M. acuminata* ssp. *microcarpa* which have pigmented foliage, such as Pandok and Zebrina or the hybrids of these clones with *M. balbisiana* were induced to tetraploidy, the colour of their foliage also increased in intensity. Microscopic observations showed that the greater colour intensity in tetraploid foliage was caused by a higher concentration of anthocyanin pigment in the vacuole of each cell rather than a doubling of the number of cells with coloured vacuoles. Horry and Jay (1988) analysed anthocyanin compositions of bracts in 59 wild and cultivated forms of *Musa* and concluded that banana classifications based on anthocyanin variation corroborated genomic classifications obtained using morphological characters. They observed varying anthocyanin patterns in the bracts of diploid, triploid, and tetraploid bananas of varying genomic constitutions.

2. 6. 2. Bunch and fruit characteristics

Vakili (1967) observed that tetraploids had smaller bunches but usually larger fruits. Tetraploids 2390 and Bodles Altafort were evaluated in Australia with 24 triploid varieties (Turner, 1984). The major defects of Bodles Altafort were excessive height (and consequently the highest losses), small bunch, short fingers and a slow ratooning speed. It is noteworthy that the ratooning rate of 2390 was similar to Robusta even though Highgate which has a very slow ratooning rate was one of the parents of this tetraploid. Hence, the author concluded that the diploid parent could strongly influence ratooning rate.

Tetraploids evaluated in Jamaica and England were reported to possess bunch size, finger weight and yield not much different from those of Valery (New and Marriott, 1976). No tetraploid was identified which did not have an inherent tendency to drop the fingers when ripe. All tetraploid fruits had weak necks. Further it was concluded that the trait was inherited from the triploid Highgate (3n female) rather than a consequence of polyploidy.

2.6.3. Fertility status

It is well known that experimentally produced tetraploid strains of diploid species show a reduced fertility as related to their diploid initial lines. This is true for the pollen as well as the seed fertility. Hence, tetraploids are associated with a negative selection value. This statement is of course valid only for sexually propagating species, while the reduction of the seed production does not influence the selection value of species with a well functioning vegetative propagation. Meiotic anomalies are considered responsible for the reduced fertility status of polyploids. Vakili, 1967 encountered a number of either female or male, or both female and male sterile plants in colchicine-induced tetraploid *M.acuminata* species, whereas, only one secondary *M.balbisiana* tetraploid plants was found to be partially female sterile. In contrary to these theories, Cheesman (1932a, b) found that "seeds freely set and germinated" in Gros Michel-derived tetraploids. He also observed that the tetraploid IC1, although having abundant pollen, "exhibited a high degree of self-incompatability". A tetraploid H6 (ABBB) recorded the highest pollen output (70,200 grains/anther) among all the cultivars compared by Apshara (2000).

2.7. Demerits of ployploidy

2.7.1. Homozygosity

Colchicine doubling and other forms of somatic doubling may be detrimental and results often have been disappointing as observed by Sanford, (1983). Heterozygosity is not increased and homozygosity is increased by having more redundant alleles. Nevertheless, it is now realized that polyploidy breeding can be more successful if a strategy is employed which builds up heterozygosity after the primary doubling. It seems clear that polysomic polyploidy (homologous genomes with random intergenomic pairing in meiosis) is likely to be successful only when it results in an increase in heterozygosity relative to related diploids (Cheesman, 1934). This requires repeated crossing cycles.

2.7.2. Weak agronomic characters

Vakili (1962) reported tetraploids as tall plants (3-5m) with drooping, weak petioles, producing fewer roots, few suckers that took longer to emerge, and slower ratooning, with smaller bunches but larger fruits.

2.7.3. Extended life cycles

Tetraploids are often inferior to their diploid ancestors because the transfer from 2n to 4n reduces in many cases the physiological capacity of the plants. This holds true with regard to:

- the speed of the ontogenic development
- the delay of flowering and ripening

Colchicine-induced tetraploids of *M. balbisiama* recorded a shooting time of 15.5 months against 13 months of the original diploids (Vakili, 1967)

2.7.4. Lower degree of firmness of fruit pulp

Baldry *et al.*(1981) compared 31 tetraploid clones with Valery for consumer acceptability in England. Two of the tetraploid clones were similar in acceptability to Valery, which even when fully ripe, was distinctively harder, while the tetraploids tended to be soft.

In summary, no tetraploid generated by the only breeding strategy employed to date, has the ideal quality, agronomic and resistance characteristics required by international commerce.

2.7.5. Instability of polyploids

Experimentally produced polyploid plants of different species have been reported to show a diverging behavior with regard to their cytological stability. Mostly, they are stable, maintaining their level of ploidy over a long number of generations. In many species, however, cytologically unstable plants are known, the off-springs of which return to the original ploidy level as reported (Hertzsch, 1951, 1959; Reddy, 1952; Vakili, 1962). Colchicine-induced tetraploids of *M. acuminata* exhibited diplo-tetraploid chimeras which was a sign of reversion to diploidy (Vakili, 1962). Out of 200 accessions, 13 were listed as tetraploids in Papua New Guinea. However, Shepherd *et al.*(1987) opined that some of these might be duplicates and some, on cytological examination, might not be tetraploids.

2.8. Ploidy assessment methods

2.8.1. Karyotype analysis

Ploidy can be assessed by chromosome counts, eventually aided by experimental metaphase arrest in the actively dividing cells. D'Amato (1952,1977) and Nagl (1978) revealed that in plant tissues consisting of non-dividing cells, their chromosome complement can be analysed under the microscope following mitotic stimulation by wounding and chemical (especially hormone treatment). Further, in the case of true polyploidy (multiplication of the chromosome number), somewhat reliable information on ploidy levels could be obtained from counting the nucleoli and/or the chromocentres (D'Amato, 1984).

Contrary to the importance of bananas and plantains both as staple food and export commodity, the knowledge of their genome at the chromosomal level remains poor (Dolezel *et al.*, 1994, 1997). It is surprising that most genotypes maintained in gene banks have unknown, a speculative or even a wrongly determined ploidy level. (Jenny *et al.*, 1997 and Horry *et al.*, 1998). One such case is the disapproval (Jenny et. al., 1997) of the tetraploidy status of the Thailand accession 'Klue Teparod', which was for long considered a tetraploid based on the works of White (1928). Detailed examinations (Horry et.al.,1998) of White's publications indicated that chromosome numbers were more often overestimated than underestimated, the actual *Eumusa* diploid samples being counted as 24 and triploids as 32 to 36. Recent works of Osuji et. al. (1996) on karyological analysis of the *Musa* accessions Klue Teparod, Pisang Jambe and '(Kluai) Ngoen' based on the novel method of chromosome counting developed by Dolezel et. al. (1998) and flow-cytometry (as per the method standardized by Dolezel et. al. 1997; 1998) confirmed the triploid status of Klue Teparod, besides revealing the triploid status of Pisang Jambe which had also been initially classified as a tetraploid, and the tetraploid status of '(Kluai) Ngoen' which had been initially classified as a triploid.

Although ploidy can be now determined also by *flow-cytometry* (Dolezel *et al.*, 1994, 1997), detailed microscopic analysis of karyotype cannot be replaced by other methods. In contrast with the situation in other economically important crops, the state of *Musa* karyology is rather poor. This is mainly due to problems with preparation of chromosome spreads and the small size of *Musa* chromosomes (1 – 2 μm, when observed at mitotic metaphase). Considering the nuclear genome size in *Musa,* an average chromosome has only about 50Mbp DNA (Dolezel *et al.*, 1994).

Difficulties in preparing good metaphase spreads are mainly due to the rigid cell wall. The tissue can be softened by maceration in strong acids or by incubation in hydrolic enzymes, and metaphase spreads can be obtained by squashing the softened tissue. In *Musa,* several authors used this technique and in some cases, metaphase spreads suitable for chromosome counting were obtained (Shephered and Santos, 1996; Osuji *et al.*, 1996). However, due to the presence of remnants of cell wall and cytoplasam, the squash technique is not suitable for high chromosome analysis as the cytoplasm may be non-specifically stained. Alternative procedures based on protoplast isolation and spreading were developed for some species to avoid this problem

(Ma *et al.*, 1996; Martin *et al.*, 1996). Dolezel *et al.* (1994) developed a novel protocol for the preparation of high quality metaphase spreads for chromosome analysis in *Musa*. This method based on protoplast isolation from root tips has several advantages including the possibility of accurate chromosome counting, for high resolution studies of the morphology and for physical mapping of *Musa* genome.

An imporved protocol for chromosome counting using a staining technique with Hoetsch colour was developed by Vandenhout (1993). It has been reported to give better results than other staining techniques with leuco-basic fuchsine, aceto-carmine or propio-carmine.

2.8.2. Cytophotometry or microdensitometry

This method is used to determine ploidy levels based on measurements of nuclear DNA content. This method has proved the validity of a commonly used method of ploidy determination (D'Amato, 1989). This is explained by the fact that each doubling of the genome - be it due to doubling of chromosome number (true polyploidy) or due to doubling of the number of chromatids within each chromosome (polyteny) – is reflected in a proportionate increase in the nuclear volume (D'Amato, 1984; Nagl, 1978). Consequently, ploidy determinations that were based on volumetric analysis of interphase nuclei have been reported to have considerable degree of reliability.

Estimations of nuclear DNA by Bennett *et al.* (1972, 1976, 1982, 1991, 1995) and Grime and Mowforth (1982) have led to the conclusion that monocotyledons as a group have genome sizes which are generally larger than those of the dicotyledons. A functional and, thereby, evolutionary role is ascribed to the total amount of DNA in a cell, based on the correlation of genome size with celluar and phenotypic characteristics such as mitotic and meiotic cell cycles, generation time, germination and phenology (Bennett, 1972; Cavalier-Smith, 1985; Grime *et al.*, 1985).

A comprehensive analysis of the evolution of genome size in *Musa acuminata* (Dolezel *et al.*, 1994; Das and Das, 1994) included the estimation of *in situ* nuclear DNA content through cytophotometry along with karyotype analysis and interphase nuclear volume (INV). Sparrow *et al.* (1975) stated that the INV was directly related to the DNA content of the nucleus at the intra-specific level. Several reports have confirmed a good correlation between INV and DNA content per nucleus (Avanzi *et al.*, 1966; Yamaguchi and Tsunoda, 1969; Katayama, 1971; Das and Mallick, 1989 a, b and c; Das and Das, 1997).

Intervarietal and interspecific variations in the nuclear DNA content have been reported in several other species (Price, 1976; Mukherjee and Sharma, 1986; Chattopadhyay and Sharma, 1990; Das and Mallick, 1993a, b). The variability in the stable content at the varietal/cultivar level might be attributed to the loss or addition of many repeats in the micro- and macro-environment of the genome during evolution (Price *et al.*, 1980; Das and Das, 1994).

2.8.3. Flow-cytometry

Determination of ploidy levels or chimeric tissues is conventionally conducted by means of microscopic chromosome counting using meristematic tissues from individual plants. Mitotic cells are arrested in metaphase, followed by DNA stained squash preparation (Dyer, 1963). Since in this way only individual plants can be screened, ploidy analysis of larger population is very time-consuming. Further, in some plant species as in the case of *Musa*, somatic chromosome counting is rather difficult. Alternatively, ploidy can be judged from the size of pollen or the number of chloroplasts in the epidermal guard cells. However, these procedures have a low level of accuracy, especially when the ploidy difference is small (*eg.* 3n Vs 4n). Moreover, ploidy screening based on pollen size requires waiting till the plantlets flower. The techniques of flow-cytometry enables rapid and precise measurements to be made on whole cells, isolated nuclei or chromosomes in a monodisperse suspension. Flow-cytometry is being extensively used in various fields of biology and medicine because of the relative ease, fast speed (>1000 particles second^{-1}), precision of measurements and sorting capabilities of modern instruments.

Since automated fluorescence methods have been introduced, (van Dilla *et al.*, 1969; Gohde and Dittrich, 1971), ploidy measurements have found a wide field of application in human oncology (Gohde *et al.*, 1979). Later, several groups of scientists have been successful in using the so called flow-cytometry in order to measue the nuclear DNA content of plant cells in a wide variety of species (Meadows, 1982; Galbraith *et al.*, 1983; Puite and Ten Brooke, 1983; Ulrich and Ulrich, 1986). More recently, flow-cytometry of nuclei stained with DNA fluorochromes has been recognized as a fast, accurate alternative to microspectrophotometry (Dolezel *et al.*, 1997).

De Laat *et al.* (1987) standardized a routine method for accurate detection of individual plants with deviating ploidy, like haploids and polyploids developed after culture of sporophyte tissues and colchicines treatments, respectively, and to calculate the percentage of plants with aberrant ploidy levels from larger plant populations. A strong correlation between DNA values determined by Feulgen microspectrophotometry and flow-cytometry has been shown by Michaelson *et al.* (1991) who devised an improved procedure for determing DNA amounts of plant nuclei adopting laser flow-cytometry.

2.8.4. Analysis of stomata

Simmonds (1948a) and Borges (1971) observed that stomata size was proportional to ploidy in banana, while stomatal density had the expected complementary relationship in Jamaican breeding programmes, stomatal densities of two month old seedlings were employed to screen the progenies ploidy-wise (Shepherd, 1968). Sathiamoorthy (1973) assessed the stomatal densities of Indian bananas of various ploidy levels and genomic constitutions. Tetraploids derived from the diploid clone SH-3362 had a mean stomatal length of 26.9μm as against 16.0μm in the diploid (Smith *et al.*, 1993).

Vandenhout *et al.*(1995) assessed the reliability of ploidy determination using stomatal measurements by correlating stomatal traits with chromosome counts in root tips of the hybrids 'Obino 1' Ewai x Calcutta 4 and in Bobby Tannap x Calcutta 4 and observed that in general, size and density of stomata, which are negatively correlated, varied according to ploidy level. Diploid hybrids had on an average 29 stomata mm^{-2} with an average size (length x width) of $1250\mu^2$, while tetraploids had on an average 15 stomata mm^{-2} with an average size of $1840\mu^2$.

In vitro derived plantlets of the diploid *M. acuminata* clone SH-3362 following treatment with anti-mitotic agents (colchicine or oryzalin) were screened for ploidy status initially based on stomatal measurements before subjecting them to flow-cytometry (van Duren *et al.*, 1996). Plantlets with stomata 29-40μm long and a density of less than 60 stomata mm^{-2} turned out to be tetraploids and plants of higher ploidy levels. Stomatal densities of $43.51mm^{-2}$, $31.08mm^{-2}$, $17.27mm^{-2}$ and $10.50mm^{-2}$ were observed for diploid, triploid, tetraploid and pentaploid hybrids of *Musa* by Apshara (2000).

2.8.5. Chloroplast density

Chloroplast density in stomatal guard cells of the sporophyte provides a means for rapid determination of ploidy state based on the postulate that chloroplast number in guard cells should increase as cell size increases in response to increased ploidy state. It is of practical importance for *Musa* breeders to determine the ploidy status of clones before they are established in field evalution plots. This could be readily achieved using chloroplast profiles from nursery seedlings. Tenkouano *et al.* (1998) in his attempt to determine ploidy level of diploids, triploids and tetraploids of *Musa* based on chloroplast characteristics, observed that the average number of chloroplasts in triploid and tetraploid was, respectively, 1.30 and 1.53 times as great as that in diploids. He recorded chloroplasts densities of 10.1, 13.1 and 15.5 in diploid cultivars (AA), triploid cultivars (AAA, AAB, ABB) and tetraploid hybrids (AAB x AA, ABB x AA) respectively.

Mattheij *et al.* (1992) claimed that ploidy assessment through chloroplast count was as consistent as that based on flow-cytometry, based on the highly reliable results he observed with his protoplast fusion-derived interspecific hybrids of potato. Further, ploidy analysis based on chloroplast counts was claimed to be even more advantageous than that based on flow-cytometry due to demerits such as sophistication and high expenses associated with flow-cytometry.

2.8.6. Pollen characteristics

Variations in pollen size owing to variations in ploidy levels have been reported in banana. Increase in the pollen volume, nucleolar volume and prophase cell volume with a corresponding increase in ploidy level has also been reported (Larter, 1935; Simmonds, 1948a; Wilson, 1948). The wide range of pollen size variation, particularly in triploids and tetraploids, was an indicative of highly aberrant male meiosis which might be due to genic disharmony (Dodds and Simmonds, 1946). Sathiamoorthy and Rao (1980) recorded mean pollen diameters of 114.6μ, 139.2μ and 157.7μ for diploid, triploid and tetraploid clones respectively.

Pollen derived from normal microsporogenesis should contain chromosome numbers that reflect the ploidy state of the sporophhyte. If this hypothesis is true, the average typical diameter should be a function of genome size (Dessauw, 1988), since both the nucleus and the cytoplasm are expected to increase as chromosome number increases. Darlington (1937) classified giant pollen grains having more than 160μ as 2n pollen. This was observed to be 1.25 times the length of haploid (n) pollen which never exceeded 128μ in non-2n pollen producers of *Musa* (Ortiz *et al.*, 1997).

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present investigations on **'*In vitro* induction of tetraploidy in diploid bananas (*Musa* spp.)'** was carried out at the Tissue Culture Laboratory of the Department of Fruit Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore during 1999-2001.

3.1 MATERIALS

3.1.1.Plant Materials

(a) Cultivars

The following diploid banana cultivars were involved in the investigations.

Table 1. Cultivars used as diploid starting materials

S.No.	Cultivar and genomic constitution	Attribute considered for selection of the cultivar
1	Sannachenkadali (AA)	Resistance to leaf spot disease (*Mycosphaerella* spp)
3	Anaikomban (AA)	Tolerance to burrowing nematode (*Radopholus similis*)
2	Kunnan (AB)	Tolerance to Panama disease (*Fusarium oxysporum* f.sp. *cubense*), leaf spot (*Mycosphaerella* spp.) and burrowing nematode (*Radopholus similis*)
4	Thattillakunnan (AB)	Tolerance to burrowing nematode (*Radopholus similis*)

(b) Donor plants

Plants of the above mentioned diploid cultivars maintained at the University Orchard served as the donor plants for the *in vitro* polyploidization experiments.

(c) Explants

Shoot tips isolated from peepers and medium sized suckers were the explants used.

3.1.2. Chemical environment for *in vitro* culture

(a) Culture medium

The mineral formulation employed was MS medium (Murashige and Skoog, 1962) (Appendix 1). The cytokinin BAP (Benzyl aminopurine), the gibberellin Gibberellic acid (GA_3) and the auxins NAA (Naphthalene acetic acid) and IBA (Indole butyric acid) were the growth regulators involved. The carbon source employed was sucrose and the gelling agent was agar. (Macro- and micro-nutrients, sucrose and agar used were of Hi-media grade. Growth regulators were of cell culture tested grade from Sigma Chemical Co., USA).

The composition of the nutrient media is detailed below.

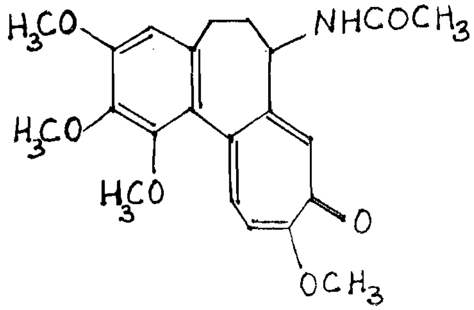
Table 2. Nutrient media used with respect to the various stages of *in vitro* culture

Stage of *in vitro* culture	Nutrient medium used		
	Strength of MS mineral formulation	**Sucrose conc. (%)**	**Growth regulators (mgl^{-1})**
Culture establishment	Full	3.0	BAP (5.0) + NAA (0.5)
Shoot proliferation	Full	3.0	BAP (5.0)
Shoot elongation	Full	3.0	GA_3 (0.5)
Rhizogenesis	Half	1.5	IBA (0.5)

(b) Anti-mitotic agents involved to induce chromosome doubling

1. Colchicine:

Chemical formula : $C_{22}H_{25}NO_6$
Molecular weight : 399.45
Chemical structure :

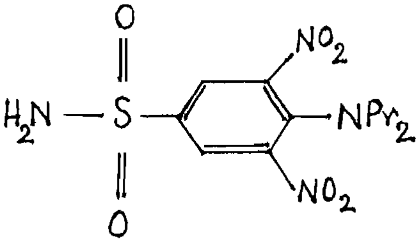


2. Oryzalin:

Chemical names – (i)3,5-dinitro-N4, N4 - dipropylsulfanilamide
(ii)4 - (dipropylamino) - 3,5 - dinitrobenzene - sulfonamide

Molecular weight : 346.36

Chemical structure :



Colchicine used was of Sigma grade and oryzalin of 'Chem Service' grade.

3.1.3. Physical environment for *in vitro* culture

The shoot tip cultures were incubated in a culture room with physical parameters maintained as furnished below:-

Temperature :25±2°C
Humidity :70 – 80 %
Light source : White fluorescent lamps
Light intensity : 2500 – 3000 lux
Photoperiod : 16 / 8 hr light / dark

3.1.4. Others

(a) Filter sterilization facility

The anti-mitotic agents namely colchicine and oryzalin were filter-sterilized using a membrane filter (Gelman Sciences) and added to the culture medium, in order to avoid destruction of their activity by heat sterilization.

(b) Glassware

Culture bottles (400 ml capacity) made of borosilicate were used for culturing the shoot tips.

(c) Medium for *ex vitro* establishment

Sterilized pot mixture made up of equal proportions of sand, red earth and FYM was the medium used for *ex vitro* establishment of the regenerated plantlets.

3.2. METHODS

3.2.1. Donor plant selection

The following criteria were considered in selection of the donor plants:

a) Trueness to type
b) Good growth rate and vigour
c) Freedom from disease and pest infestation
d) Desirable fruit characteristics
e) Desirable sucker appearance and health

3.2.2. Explant preparation

(a) Isolation of explant tissue:

Fig.3. Steps adopted in isolation of the explant tissue

Collection of healthy peepers and suckers
from field-grown donor plants

↓

Removal of adhering soil by
Washing with running water

↓

Soaking peepers and suckers in fungicidal
Solution (0.5% carbendazim) for 2-3 hours to
reduce the microbial load.

↓

Washing with a liquid detergent
(Tween – 20) in running water.

↓

Trimming the peepers and suckers.

↓

Isolation of shoot tips by removing the surrounding
leaf sheaths one by one until 2 – 3 leaf sheaths and a
portion of the rhizomatous base remained intact with
the shoot tip (length : 2.5 - 3.0 cm ; diameter : 1.5 - 2.0 cm)

(b) Pre-treatment of explants

The isolated shoot tips were treated with an anti-oxidant mixture of 50 mgl^{-1} of each of the anti-oxidants, viz., citric acid and ascorbic acid for 10 –15 minutes in order to prevent browning of the explants.

(c) Surface sterilization of explants

The following steps were followed to surface sterilize the explants:

- Rinsing the shoot tips with distilled water
- Treatment with 70% ethanol for 30 – 45 seconds
- Treatment with 0.1% $HgCl_2$ for 10 minutes

- Rinsing 3 times with double distilled water (inside the aseptic environment provided by a laminar airflow chamber).

3.2.3.Preparation of culture medium

(A) Preparation of stock solutions

(a) Stock solutions of macro- and micro-elements and organic constituents

Stock solutions of macro and microelements were prepared by dissolving appropriate quantities of each element in respect of the MS medium. For stock solution of ferrous sulphate, required quantities of FeSo$_4$ .7H$_2$O and Na$_2$EDTA in respect of the Murashige and Skoog medium were dissolved separately; the Na$_2$EDTA solution was slightly warmed in order to aid the completion of the chelation reaction and was added gently to the FeSO$_4$ solution. The final volume was made up with distilled water. Stock solution of the organic constituents namely the vitamins and amino acids were maintained separately.

(b) Stock solutions of growth regulators

Auxins : Auxins were dissolved in 2 to 3ml of 1N NaOH, heated slightly and made up to the final volume with distilled water.

Cytokinins : Cytokinins were dissolved in 1N HCl, heated slightly and made up to the final volume with distilled water.

(d) Stock solutions of anti-mitotic agents

Molar concentrations of the anti-mitotic agents were prepared by dissolving the required quantity of colchicine/oryzalin initially in a 2% (v/v) DMSO (Dimethyl sulfoxide) solution and making upto the final volume with distilled water.

(B) Preparation of culture medium

The following steps were adopted to prepare the culture medium :

- Mixing up of stock solutions of macro- and microelements, iron, organic constituents.
- Addition of sucrose
- Addition of 0.8% of melted agar to the basal medium.
- Addition of the required growth regulators.
- Autoclaving the medium at a temperature of 121°C and a pressure of 15 psi for 20 minutes.
- Allowing the medium to cool down to 45 – 50°C
- Addition of filter sterilized solutions of colchicine / oryzalin.
- Homogenization
- Distribution to sterilized culture vessels.

3.2.4. Modes of treatment with anti-mitotic agents

The following two methods were adopted to treat the shoot tips with the anti-mitotic agents.

(i) Soaking the shoot tips in a solution of the anti-mitotic agent and placing them in a gyratory shaker operating at 60 rpm for the required durations (van Duren *et al.*,1996)

(ii) Inclusion of filter sterilized solutions of either of the anti-mitotic agent in the culture medium on which untreated shoot tips were placed for the specified durations.

3.2.5. Inoculation and incubation of explants

The anti-mitotic agent treated shoot tips were washed with sterile water and inoculated on culture establishment medium contained in sterilized culture bottles and incubated in the culture room.

3.2.6. Subculture of established primary cultures

Subculture was done at monthly intervals by transferring the primary cultures to shoot proliferation medium devoid of anti-mitotic agent. The cultures were subjected to three subcultures, i.e., each shoot tip was propagated for three vegetative cycles (V_1, V_2 and V_3), carefully numbering the progenies at each subculture.

3.2.7. Experimental details

Details of the treatments are furnished below.

(I) *In vitro* induction of tetraploidy

(a) Sensitivity to anti-mitotic agents

The details of the experiments conducted to assess the sensitivity of the shoot tip cultures to colchicine and oryzalin are presented in Appendices 2 and 3. Among the treatments, only those which resulted in survival rates of around 50% (Appendices 4 and 5) and above were considered to assess the efficiency of the anti-mitotic agents in inducing tetraploidy under *in vitro* conditions.

(b) Effect of anti-mitotic agents on shoot regeneration:

Among the shoot tips which survived following treatment with anti-mitotic agents, those which responded to regeneration by way of turning green (referred to as shoot tip greening hereafter) followed by a remarkable increase in size (Plate 1) were identified as the cultures responding to shoot regeneration and were subjected to subsequent sub-culturing for three vegetative cycles. Response to greening was recorded up to 30 days after inoculation.

The response of the cultures to shoot proliferation during the three vegetative cycles on the shoot proliferation medium was recorded in order to analyse the influence of the anti-mitotic agents on shoot proliferation. The time taken for induction of multiple shoots, number of multiple shoots produced per culture, the length of the microshoots and their fresh weights were recorded.

(c) Effect of anti-mitotic agents on *in vitro* rhizogenesis

Microshoots regenerated in V_3 were transferred to rooting medium to induce *in vitro* rhizogenesis.

(d) *Ex vitro* establishment

Fully developed plantlets were removed from the culture vessels and washed gently in running water to remove the adhering nutrient medium completely, without causing any damage to the roots. The roots were immersed in a fungicidal solution (0.5% carbendazim) for an hour after which the plantlets were transferred to pots filled with sterilized pot mixture. The plantlets were kept in a mist chamber and irrigated with distilled water on alternate days for a period of two weeks followed by fertigation with a 1/10 MS nutrient solution at a frequency of two times a week.

(II) Verification of ploidy status of the regenerants

(a) Estimation of stomatal characters

The number of stomata mm^{-2} has been reported to be proportionate to the ploidy level upto pentaploidy (Simmonds, 1948a and b; Sathiamoorthy, 1973). The standard stomatal density values for different ploidy levels as assessed by Sathiamoorthy (1973) and stomata size as per the reports of Vandenhout *et al.* (1995) have been taken to assess the ploidy status of the regenerated plantlets. The mean stomatal density values and stomata size for the various ploidy levels are as follows:

Ploidy	**Stomatal density No. of stomata mm^{-2}**	**Stomata size (Length x Breadth μ^2)**
2n	40.00 - 50.00	1250.00
4n	9.00 - 15.20	1840.00
	(Sathiamoorthy, 1973)	(Vandenhout *et al.*, 1995)

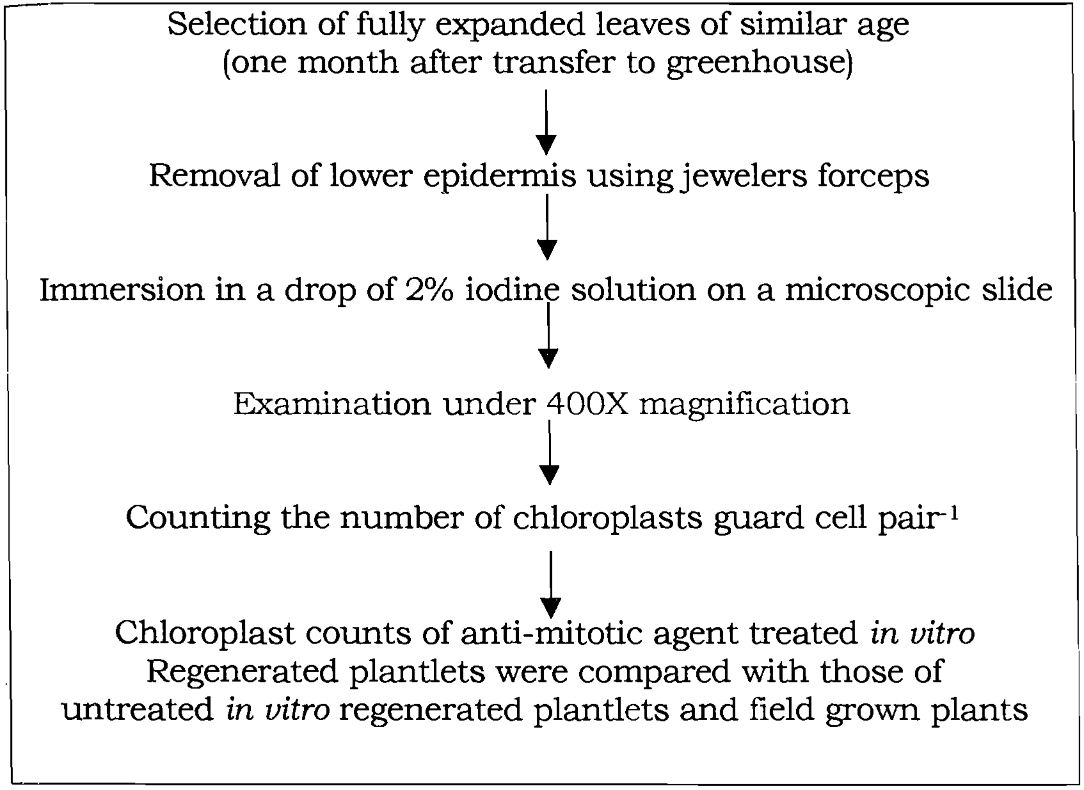
Leaves of three to four month old plants were sampled in the early morning hours to analyze the stomatal density and stomata size. Measurements were made from the upper surface of the lamina of the third leaf from top.

(b) Estimation of chloroplast density in stomatal guard cells

Chloroplast density in stomatal guard cells can be adopted as one of the means for rapid determination of ploidy state based on the postulate that chloroplast number in guard cells should increase as cell size increases in response to increased ploidy level. This technique has been used to differentiate between diploid and tetraploid accessions of several species, including potato (Cardi *et al.*, 1993) and watermelon (Compton, *et al.*,1996; McCuistion and Elmstrom, 1993).

The procedure of Compton *et al.*, (1996) was used for sample preparation with a slight modification as adopted for *Musa* spp. by Tenkouano *et al.*, (1998). The procedure is detailed below.

Fig. 4. Procedure for estimation of chloroplast density in stomatal guard cells



(c) Estimation of somatic chromosome number

Counting and characterization of somatic chromosomes of root tips is the oldest and most accurate method of ploidy assessment. Following the preliminary ploidy screening based on stomata and chloroplast analyses, the *in vitro* regenerated plantlets were subjected to further assessment of ploidy (van Duren *et al.* 1996) based on somatic chromosome counting for a confirmation of their ploidy status..

The procedure adopted for counting the somatic chromosomes of root tips is detailed below.

Fig. 5. Procedure for estimation of somatic chromosome numbers in root tip cells

Collection of young fresh root tips

↓

Shock treatment with the pretreatment chemical (0.02M 8-Hydroxy quinoline) at 0-2°C for 5 minutes for better scattering and clarification of chromosomes)

↓

Pretreatment with 0.02 M 8-Hydroxy quinoline solution or saturated paradicholrobenzene or a mixture of these two at 15°C for 2- 3h

↓

Fixation in Clarke's fluid (1:3 of acetic acid : ethanol mixture) at room temperature for 12 - 18h

↓

Hydrolysis with 5% HCl for 2min and 9% HCl for 4min

↓

Rinsing with 70% ethanol

↓

Staining with 2% aceto-orcein

↓

Chromosome squash preparations in 45% acetic acid.

(III) Evaluation of *in vitro* induced tetraploids

The *in vitro* induced tetraploids were transferred to *ex vitro* environment and the various parameters related to the ploidy status of the plants were analysed.

3.2.8. Observations

Table 3. Details of observations recorded

Observation	**Criteria**
A) *In vitro* phase	
Per cent survival	Number of shoot tips surviving during the culture establishment phase expressed in percentage. Observations on survival were recorded up to 45 days after inoculation.
Per cent contamination	Number of cultures which succumbed to microbial contamination (bacterial and fungal) expressed in percentage.
Per cent mortality	Number of shoot tips which succumbed to phytotoxicity resulting from treatment with supra-optimal concentrations or treatment durations with anti-mitotic agents associated with a characteristic blackening of the central growing point (Plate 2) expressed in percentage.
Intensity of phytotoxicity of anti-mitotic agent (Plates 2 to 4)	Scoring: 0 - Non-toxic : Shoot tip explant not subjected to blackening caused by the anti-mitotic agent 1 - Less toxic : 3rd and surrounding leaf sheaths affected 2 - Moderately toxic : 1st and 2nd sheaths affected; meristem unaffected 3 - Highly toxic : Meristem affected
Per cent response to regeneration	Number of shoot tips responding to greening (Plate 1) expressed in percentage.
Days taken for greening	Mean number of days from the day of inoculation up to shoot tip greening.
Days for first leaf emergence	The mean number of days from inoculation till the emergence of the first leaf.
Per cent response to shoot proliferation	Number of shoot tip cultures regenerating multiple shoots.
	Contd...

Observation	**Criteria**
Days for multiple shoot bud differentiation	The mean number of days taken from the day of inoculation till differentiation of multiple shoot buds.
Number of multiple shoots per culture	The mean number of adventitious shoot initials/microshoots regenerated from a single shoot tip.
Length of microshoots	Mean length of the microshoots expressed in centimetres.
Number of leaves	Mean number of leaves per microshoot
Weight of microshoots	Mean fresh weight of the microshoots regenerated from a shoot tip expressed in grams.
Per cent response to rhizogenesis	The number of microshoots which responded to rhizogenesis on transfer to rooting medium expressed in percentage.
Days taken for rooting	The mean number of days from transfer to rooting medium up to root initiation.
Number of roots per plantlet	The mean number of roots per plantlet.
Length of roots	The mean length of the roots of a plantlet expressed in centimetres.
Total *in vitro* duration	Total number of days from the day of inoculation of the explant up to transfer of well-developed plantlet to *ex vitro* environment (Primary culture phase + three vegetative cycles + rooting phase).
B)Ploidy assessment	
Stomatal density	Measured microscopically and expressed as number of stomata mm^{-2}
Size of stomata	Area of stomata expressed in μ^2 (Length x Breadth)
Chloroplast density in stomatal guard cells	Expressed as number of chloroplasts per guard cell pair.
Somatic chromosome counts	Expressed as number of chromosomes per root tip cell.
	Contd..

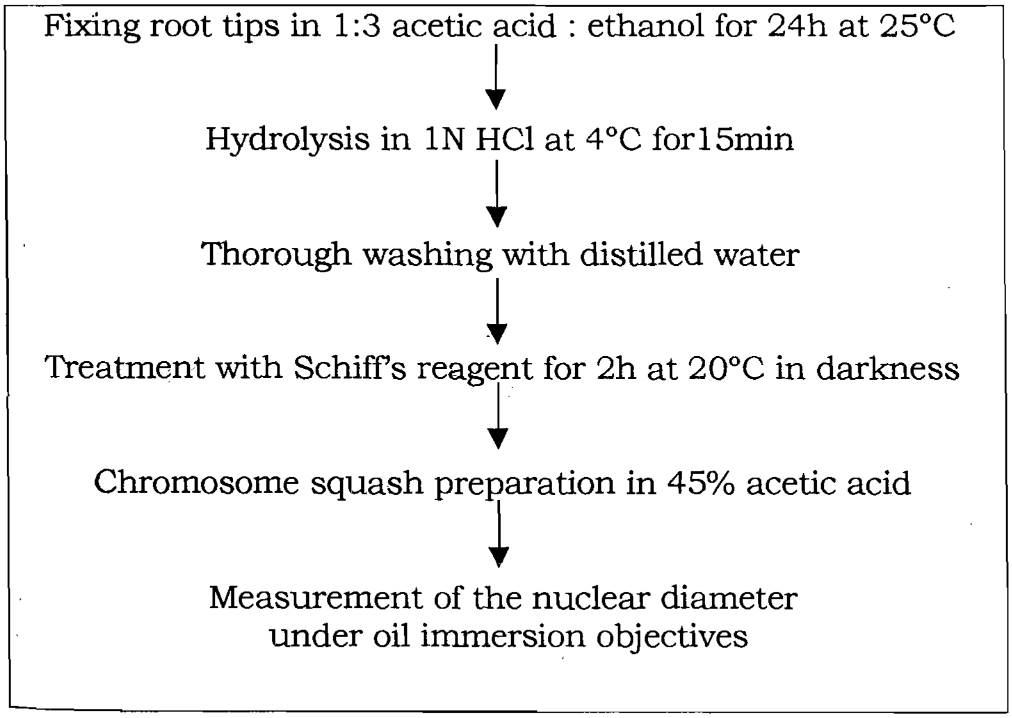
Observation	**Criteria**
C) *Ex vitro* phase	
(a) Morphological parameters:	
Plant height	The height from the base of the plant up to the angle between the youngest first and second leaf axils expressed in cm.
Pseudostem girth	The girth of the pseudostem was measured at half of its length at transfer to *ex vitro* environment and at 5cm and 10cm above the ground level two months and four months respectively after transfer to *ex vitro* environment
(b) Physiological parameters	
Leaf area	Calculated by multiplying the product of the leaf length and breadth by the factor 0.80 (Murray, 1960) and expressed in m^2.
Phyllochron	The time interval between the emergence of successive leaves expressed in days.
Leaf length-width ratio (LWR)	The ratio between the leaf length and breadth.
Leaf lamina weight	The fresh weight of the lamina of the third leaf from top expressed in grams.
Percentage dry weight of petiole	Calculated based on the fresh and dry weights of 10cm length of petiole
Photosynthetic efficiency	Estimated using photosynthesis yield analyser (MINI- PAM) portable fluorometer and expressed in quantum units.
Crop growth rate	Expressed in cm day^{-1}
(c) Biochemical parameters	
Chlorophyll content of leaves	Spectrophotometric analysis of total chlorophyll and expression in mgg^{-1} of leaf tissue.
	Contd..

Observation	Criteria
Anthocyanin pigmentation in leaves and petioles of the cv. Sannachenkadali	Expressed in mg100g^{-1} of leaf tissue (as per the method of Ranganna, 1976).
(d)<u>Cytogenetic parameters</u>	
* Interphase nuclear volume (INV)	Estimated as per the method of Das and Das (1997) and expressed in μm^3

*** Estimation of interphase nuclear volume (INV)**

For INV estimation, the method described by Das and Das (1997) was adopted. The procedure is detailed below.

Fig. 6. Procedure for estimation of INV



Fixing root tips in 1:3 acetic acid : ethanol for 24h at 25°C

↓

Hydrolysis in 1N HCl at 4°C for15min

↓

Thorough washing with distilled water

↓

Treatment with Schiff's reagent for 2h at 20°C in darkness

↓

Chromosome squash preparation in 45% acetic acid

↓

Measurement of the nuclear diameter
under oil immersion objectives

Statistical analysis

All the *in vitro* experiments were laid out following factorial Completely Randomised Design. The data were analysed as per the methods described by Panse and Sukhatme (1984). The effects of the two anti-mitotic agents were analysed separately and comparison between the two was made using 't' test. The *ex vitro* phase consisted of documentation of the various characters which reflected the ploidy status.

EXPERIMENTAL RESULTS

CHAPTER IV

EXPERIMENTAL RESULTS

The data pertaining to the various investigations conducted to analyse the efficiency of *in vitro* methods of inducing tetraploidy in four diploid clones of banana, *viz.*, Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan using the anti-mitotic agents colchicine and oryzalin are presented below.

4.1. *IN VITRO* INDUCTION OF TETRAPLOIDY

4.1.1. Effect of anti-mitotic agents on *in vitro* regeneration

4.1.1.1. Primary culture phase

4.1.1.1.1. Time taken for greening of shoot tips (Tables 4 and 5; Fig. 7)

➢ Effect of colchicine: Highly significant variations existed among the cultivars and the treatments (Table 4). Among the cultivars, Anaikomban recorded the earliest response (14.13d after inoculation) to shoot tip greening, while Kunnan recorded the most delayed response (15.36d). Among the treatments, C1 (2.5mM for 12h) led to the earliest (9.00d) shoot tip greening, whereas C5 (2.5mM for 36h) resulted in the most delayed response (26.90d).

➢ Effect of oryzalin: Treating shoot tips with oryzalin resulted in highly significant variations among the cultivars and the treatments (Table 5). Among the cultivars, Sannachenkadali was found to require the least duration (9.14d) for greening, while Thattillakunnan took the longest time (10.23d). Among the treatments, O1 (10μM for 3d) resulted in the earliest greening (7.20d), while the most delayed response (15.24d) was recorded by the shoot tips subjected to the treatment O4 (40μM for 3d). The treatments involving exposure of shoot tips to lower concentrations for longer periods, namely, O5 and O6 (10μM and 20μM for 6d each) took comparatively lesser time (8.88d and 9.79d respectively)

Table 4. Effect of colchicine on the time taken **(days after inoculation) for greening of shoot tips**

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	4.25		4.13		4.38		4.75		**4.38**
C1 (2.5mM for 12h)	8.59	102.00	8.67	110.06	9.63	120.00	9.13	92.11	**9.00**
C2 (5.0mM for 12h)	11.67	174.59	11.75	184.85	12.84	193.37	12.25	157.89	**12.13**
C3 (7.5mM for 12h)	14.50	241.18	14.25	245.45	16.17	269.49	15.75	231.58	**15.17**
C4 (2.5mM for 24h)	20.00	370.59	19.84	380.85	21.84	399.09	21.50	352.63	**20.79**
C5 (2.5mM for 36h)	26.59	525.53	26.17	534.30	27.34	524.80	27.50	478.95	**26.90**
Mean	**14.27**		**14.13**		**15.36**		**15.15**		**14.73**

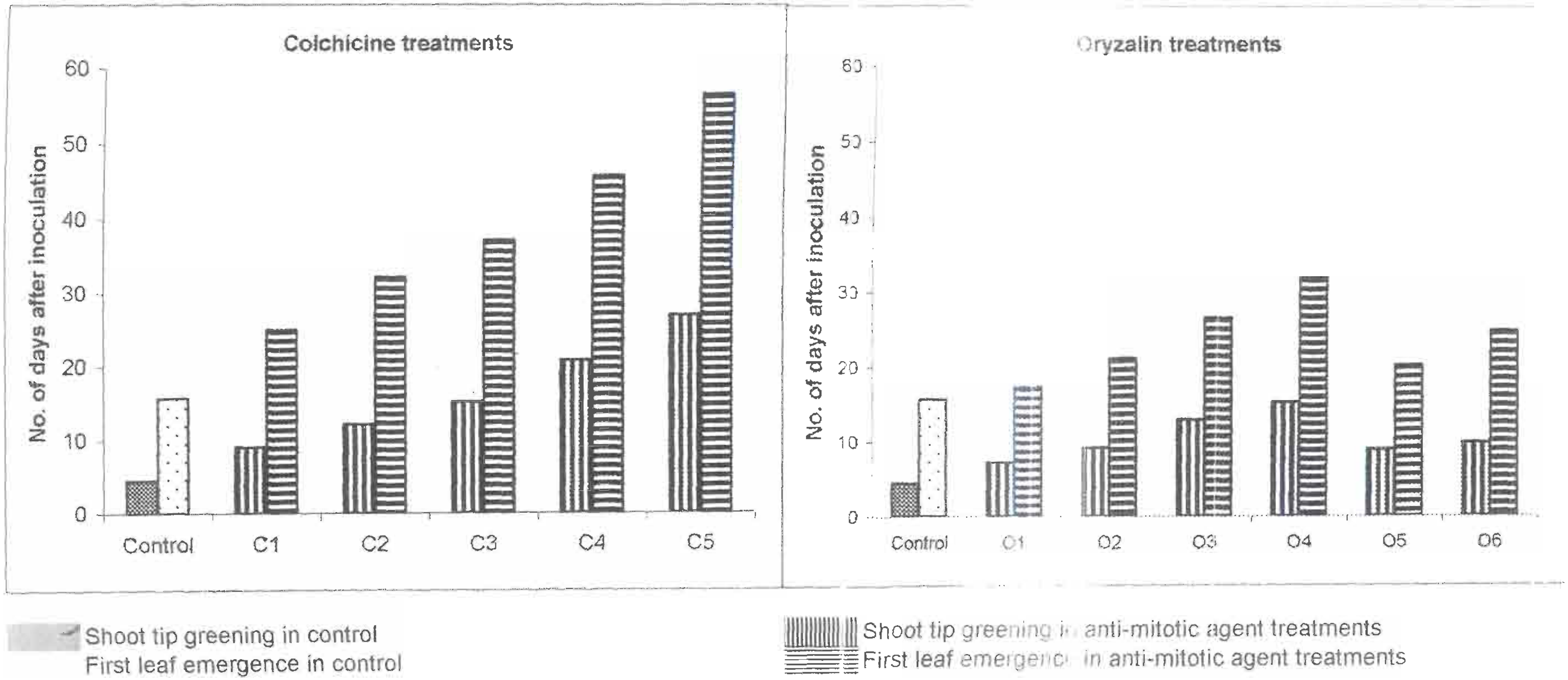
	SEd	CD (P=0.05)
Cultivar	0.158	0.327**
Colchicine	0.194	0.401**
Cultivar X Colchicine	0.388	N S

Table 5. Effect of oryzalin on the time taken (days after inoculation) for greening of shoot tips

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	4.25		4.13		4.38		4.75		**4.38**
O1 (10µM for 3d)	6.84	60.82	7.11	72.24	7.25	65.71	7.63	60.53	**7.20**
O2 (20µM for 3d)	8.63	102.94	8.59	108.12	9.67	121.03	9.88	107.89	**9.19**
O3 (30µM for 3d)	11.67	174.59	12.50	203.03	13.84	216.23	13.67	187.68	**12.92**
O4 (40µM for 3d)	14.59	243.18	15.17	267.64	15.59	256.23	15.63	228.95	**15.24**
O5 (10µM for 6d)	8.50	100.00	8.67	110.06	9.17	109.60	9.17	93.05	**8.88**
O6 (20µM for 6d)	9.51	123.76	9.17	122.18	9.63	120.00	10.88	128.95	**9.79**
Mean	**9.14**		**9.33**		**9.93**		**10.23**		9.66

	SEd	CD (P=0.05)
Cultivar	0.159	0.326**
Oryzalin	0.211	0.432**
Cultivar X Oryzalin	0.421	N S

Fig 7. Delay in the response to *in vitro* regeneration in anti-mitotic agent treated cultures



for greening than the treatment O4 (40μM for 3d). Colchicine Vs oryzalin: The data (Tables 4 and 5; Fig. 7) when subjected to 't' test revealed that the two anti-mitotic agents varied significantly. Both delayed the process of shoot tip greening, the degree of delay being higher with colchicine (an overall average of 14.73d for greening) than with oryzalin (an overall average of 9.66d for greening).

The corresponding diploid took lesser time (4.38d) for greening than the colchicine and oryzalin treatments.

4.1.1.1.2. Time taken for emergence of first leaf (Tables 6 and 7; Fig.7)

- Effect of colchicine: There existed highly significant variations among the cultivars and treatments (Table 6). Among the cultivars, Sannachenkadali expressed the earliest emergence (34.19d after inoculation) of the first leaf and Thattillakunnan expressed the most delayed emergence (36.46d). Among the treatments, C1 (2.5mM for 12h) led to the earliest emergence (24.97d) of first leaf, whereas, C5 (2.5mM for 36h) resulted in the most delayed emergence (56.48d).
- Effect of oryzalin: Treating shoot tips with oryzalin resulted in highly significant variations among the cultivars and the treatments (Table 7). Among the cultivars, Sannachenkadali was found to require the least duration (21.80d) for emergence of the first leaf and Thattillakunnan recorded the most delayed emergence (23.11d). Among the treatments, O1 (10μM for 3d) resulted in the earliest emergence of first leaf (17.23d), while the most delayed response (31.73d) was exhibited by the shoot tips treated with O4 (40μM for 3d). The treatments involving exposure of shoot tips to lower concentrations for longer periods, namely, O5 (10μM for 6d) and O6 (20μM for 6d) took comparatively lesser time

Table 6. Effect of colchicine on the time taken (days after inoculation) for emergence of first leaf

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	14.21		15.23		16.27		16.89		**15.65**
C1 (2.5mM for 12h)	24.25	70.71	23.81	56.39	26.19	60.99	25.62	51.70	**24.97**
C2 (5.0mM for 12h)	30.70	116.12	30.88	102.82	33.14	103.75	33.62	99.11	**32.09**
C3 (7.5mM for 12h)	36.26	155.23	37.07	143.48	37.40	129.91	37.39	121.41	**37.03**
C4 (2.5mM for 24h)	44.28	211.72	44.93	195.11	46.19	183.95	47.41	180.75	**45.70**
C5 (2.5mM for 36h)	55.45	290.36	55.73	266.01	56.92	249.95	57.82	242.43	**56.48**
Mean	**34.19**		**34.61**		**36.02**		**36.46**		**35.32**

	SEd	CD (P=0.05)
Cultivar	0.324	0.670**
Colchicine	0.397	0.820**
Cultivar X Colchicine	0.795	N S

Table 7. Effect of oryzalin on the time taken (days after inoculation) for emergence of first leaf

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	14.21		15.23		16.27		16.89		**15.65**
O1 (10μM for 3d)	16.82	18.41	17.20	12.97	18.00	10.64	16.92	0.21	**17.23**
O2 (20μM for 3d)	21.26	49.67	20.78	36.49	21.69	33.35	20.73	22.77	**21.12**
O3 (30μM for 3d)	25.67	80.71	26.25	72.41	26.88	65.26	27.06	60.26	**26.47**
O4 (40μM for 3d)	30.89	117.46	31.34	105.81	31.75	95.17	32.94	95.08	**31.73**
O5 (10μM for 6d)	19.57	37.73	18.77	23.25	20.80	27.88	21.42	26.86	**20.14**
O6 (20μM for 6d)	24.19	70.26	23.97	57.41	24.74	52.11	25.80	52.77	**24.67**
Mean	**21.80**		**21.93**		**22.87**		**23.11**		**22.43**

	SEd	CD (P=0.05)
Cultivar	0.288	0.591**
Oryzalin	0.381	0.781**
Cultivar X Oryzalin	0.763	N S

(20.14d and 24.67d respectively) than O4 (40µM for 3d) which involved the highest concentration of oryzalin.

➢ Colchicine Vs oryzalin: Subjecting the data (Table 6 and 7; Fig. 7) to 't' test revealed that colchicine and oryzalin varied significantly in their effect on the time required for emergence of the first leaf. Both caused a delay in the process of emergence of first leaf, the degree of delay being higher with colchicine (an overall average of 35.32d) than with oryzalin (an overall average of 22.43d).

The corresponding diploid took lesser time (15.65d) than the colchicine and oryzalin treatments, for emergence of the first leaf.

4.1.1.1.3. Response to multiple shoot regeneration (Tables 8 and 9)

➢ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interaction effects (Table 8). Among the cultivars, Sannachenkadali recorded the highest percentage (24.86) and Kunnan the lowest percentage (20.81) of response. Among the treatments, C1 (2.5mM for 12h) resulted in the highest response (30.13%) and C5 (2.5mM for 36h) the lowest (3.12%). The values for the interaction effect ranged between 2.47% for the shoot tips of Kunnan treated with C5 (2.5mM for 36h) and 32.21% for those of Anaikomban treated with C1 (2.5mM for 12h).

➢ Effect of oryzalin: There existed highly significant variations among the cultivars, the treatments and their interactions (Table 9). Among the cultivars, Anaikomban recorded the highest response (40.68%) to multiple shoot regeneration and Kunnan the lowest (34.35%). Among the treatments, O1 (10µM for 3d) recorded the highest percentage of response (39.91), while O4 (40µM for 3d) recorded the lowest (26.87). The treatments involving the lowest

Table 8. Effect of colchicine on the response (%) to multiple shoot regeneration in the primary culture

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	57.26 (49.17)		59.23 (50.31)		51.15 (45.65)		53.37 (46.93)		**55.25**
C1 (2.5mM for 12h)	31.52 (34.15)	-44.96	32.21 (34.57)	-45.62	27.48 (31.61)	-46.27	29.32 (32.78)	-45.06	**30.13**
C2 (5.0mM for 12h)	26.38 (30.90)	-53.93	28.51 (32.27)	-51.86	24.29 (29.52)	-52.52	24.22 (29.47)	-54.63	**25.85**
C3 (7.5mM for 12h)	20.19 (26.70)	-64.74	19.45 (26.16)	-67.16	17.44 (24.68)	-65.91	18.47 (25.45)	-65.39	**18.89**
C4 (2.5mM for 24h)	9.53 (17.98)	-83.36	6.20 (14.41)	-89.53	2.07 (8.26)	-95.96	2.17 (8.47)	-95.93	**4.99**
C5 (2.5mM for 36h)	4.27 (11.91)	-92.55	3.19 (10.28)	-94.61	2.47 (9.04)	-95.17	2.55 (9.18)	-95.22	**3.12**
Mean	**24.86**		**24.80**		**20.81**		**21.68**		**23.04**

	SEd	CD (P=0.05)
Cultivar	0.078	0.161**
Colchicine	0.095	0.197**
Cultivar X Colchicine	0.191	0.394**

Numbers within parentheses are arc-sine transformed values

Table 9. Effect of oryzalin on the response (%) to multiple shoot regeneration in the primary culture

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	57.26 (49.17)		59.23 (50.31)		51.15 (45.65)		53.37 (46.93)		**55.25**
O1 (10µM for 3d)	43.26 (41.12)	-24.44	42.33 (40.58)	-28.54	37.27 (37.62)	-27.14	36.79 (37.33)	-31.08	**39.91**
O2 (20µM for 3d)	40.22 (39.35)	-29.76	41.15 (39.90)	-30.53	33.16 (35.15)	-35.17	34.18 (35.77)	-35.96	**37.17**
O3 (30µM for 3d)	34.32 (35.86)	-40.06	36.18 (36.97)	-38.92	28.28 (32.12)	-44.72	27.31 (31.50)	-48.84	**31.52**
O4 (40µM for 3d)	31.34 (34.04)	-45.26	28.26 (32.11)	-52.29	23.53 (29.01)	-53.99	24.35 (29.56)	-54.38	**26.87**
O5 (10µM for 6d)	40.21 (39.35)	-29.77	39.76 (39.09)	-32.87	35.26 (36.42)	-31.06	34.52 (35.98)	-35.32	**37.44**
O6 (20µM for 6d)	37.72 (37.89)	-34.12	37.88 (37.98)	-36.05	31.81 (34.33)	-37.81	31.85 (34.35)	-40.33	**34.81**
Mean	**40.62**		**40.68**		**34.35**		**34.62**		**37.57**

	SEd	**CD (P=0.05)**
Cultivar	0.162	0.332**
Oryzalin	0.214	0.439**
Cultivar X Oryzalin	0.428	0.877**

Numbers within parentheses are arc-sine transformed values

concentrations for longer duration, namely, O5 (10μM for 6d) and O6 (20μM for 6d) recorded higher rates of response (37.44% and 34.81% respectively) than the treatment O4 (40μM for 3d) involving the highest concentration of oryzalin which recorded 26.87% response.

➢ Colchicine Vs oryzalin: The data (Tables 8 and 9) when subjected to 't' test revealed that colchicine and oryzalin varied significantly from each other. Colchicine caused a higher degree of decline in the rate of response to multiple shoot regeneration with an overall average of 23.04%) than oryzalin (an overall average of 37.57%).

The corresponding diploid recorded a higher rate of response (55.25%) than the colchicine and oryzalin treatments.

4.1.1.1.4. Number of microshoots regenerated (Tables 10 and 11; Fig. 8)

➢ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interaction effects (Table 10). Among the cultivars, Sannachenkadali regenerated the highest number of shoots per culture (2.28), while Thattillakunnan regenerated the lowest (1.55). Among the treatments, C1 (2.5mM for 12h) led to the highest number of shoots per culture (1.91), and C5 (2.5mM for 36h) resulted in the lowest (1.10). The values for the interaction effect ranged between 1.05 shoots per culture for the shoot tips of Thattillakunnan treated with C5 (2.5mM for 36h) and 2.78 shoots per culture for those of Sannachenkadali treated with C1 (2.5mM for 12h).

➢ Effect of oryzalin: Treating shoot tips with oryzalin caused significant variations among the cultivars and the treatments (Table 11). Among the cultivars, Sannachenkadali regenerated the highest number of shoots per culture (4.67) and Thattillakunnan

Table 10. Effect of colchicine on the number of microshoots regenerated per shoot tip in the primary culture

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	4.91		4.49		3.20		3.27		**3.97**
C1 (2.5mM for 12h)	2.78	-43.32	2.09	-53.45	1.39	-56.56	1.39	-57.43	**1.91**
C2 (5.0mM for 12h)	2.09	-57.39	2.23	-50.45	1.50	-53.28	1.36	-58.35	**1.79**
C3 (7.5mM for 12h)	1.53	-68.91	1.76	-60.80	1.32	-58.91	1.11	-66.16	**1.43**
C4 (2.5mM for 24h)	1.28	-74.01	1.24	-72.49	1.16	-63.75	1.11	-66.16	**1.19**
C5 (2.5mM for 36h)	1.09	-77.88	1.18	-73.83	1.07	-66.56	1.05	-67.84	**1.10**
Mean	**2.28**		**2.16**		**1.61**		**1.55**		**1.90**

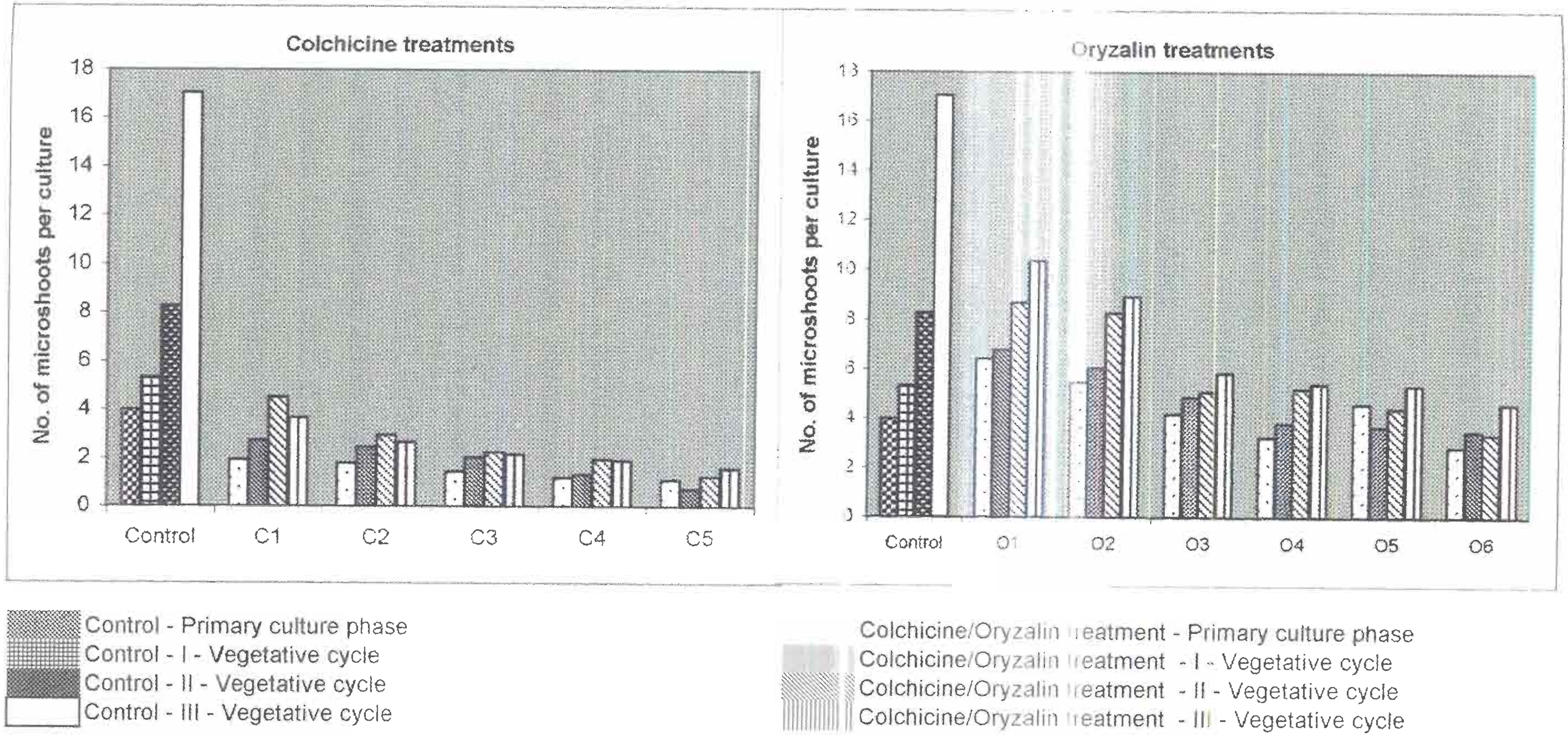
	SEd	CD (P=0.05)
Cultivar	0.105	0.217**
Colchicine	0.129	0.265**
Cultivar X Colchicine	0.257	0.531**

Table 11. Effect of oryzalin on the number of microshoots regenerated per shoot tip in the primary culture

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	4.91		4.49		3.20		3.27		**3.97**
O1 (10µM for 3d)	6.57	33.94	6.10	35.86	6.48	102.34	6.47	98.01	**6.40**
O2 (20µM for 3d)	5.37	9.48	5.79	28.95	5.11	59.69	5.55	69.98	**5.46**
O3 (30µM for 3d)	4.49	-8.46	3.96	-11.92	4.29	34.06	3.94	20.52	**4.17**
O4 (40µM for 3d)	3.33	-32.21	3.38	-24.72	3.10	-3.13	3.15	-3.52	**3.24**
O5 (10µM for 6d)	4.94	0.61	4.67	4.01	4.54	41.88	4.12	26.03	**4.57**
O6 (20µM for 6d)	3.08	-37.21	3.45	-23.16	2.68	-16.41	2.06	-37.06	**2.82**
Mean	**4.67**		**4.55**		**4.20**		**4.08**		**4.37**

	SEd	CD (P=0.05)
Cultivar	0.174	0.356**
Oryzalin	0.230	0.470**
Cultivar X Oryzalin	0.459	N S

Fig 8. Effect of anti-mitotic agents on multiple shoot regeneration



the lowest (4.08). Among the treatments, O1 (10μM for 3d), O2 (20μM for 3d) and O3 (30μM for 3d) regenerated more number of shoots per culture than the corresponding diploid (3.97 shoots), the values being 6.40, 5.46 and 4.17 respectively. The lowest number of shoots (2.82) was regenerated by O6 (20μM for 6d).

➤ Colchicine Vs oryzalin: 't' test of the data (Tables 10 and 11; Fig. 8) revealed that colchicine and oryzalin were statistically different from each other. Colchicine caused a suppression in the number of multiple shoots regenerated per culture (an overall average of 1.90 shoots per culture), whereas oryzalin caused an increase in the same (an overall average of 4.37 shoots per culture).

The number of shoots regenerated by the corresponding diploid (3.97) was higher than those of the colchicine treatments and lower than those of the oryzalin treatments O1, O2 and O3.

4.1.1.2. First vegetative cycle

4.1.1.2.1. Response to multiple shoot regeneration (Tables 12 and 13)

➤ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interactions (Table 12). Among the cultivars, Sannachenkadali recorded the highest response (28.70%), while Kunnan recorded the lowest (23.64%). Among the treatments, C1 (2.5mM for 12h) recorded the highest response (31.89%) and C5 (2.5mM for 36h) the lowest (5.60%). The values for the interaction effect ranged from 3.52% for the shoot tips of Kunnan treated with C5 (2.5mM for 36h) to 34.18% for those of Sannachenkadali treated with C1 (2.5mM for 12h).

➤ Effect of oryzalin: Treating shoot tips with oryzalin resulted in highly significant variations among the cultivars, the treatments and their interaction effects (Table 13).

Table 12. Effect of colchicine on the response (%) to multiple shoot regeneration in the first vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	63.22 (52.66)		61.23 (51.48)		52.27 (46.29)		53.42 (46.96)		**57.53**
C1 (2.5mM for 12h)	34.18 (35.77)	-45.94	32.31 (34.64)	-47.23	30.40 (33.45)	-41.84	30.67 (33.65)	-42.60	**31.89**
C2 (5.0mM for 12h)	30.36 (33.43)	-51.97	31.28 (34.00)	-48.92	26.15 (30.75)	-49.97	24.23 (29.48)	-54.65	**28.00**
C3 (7.5mM for 12h)	25.27 (30.17)	-60.03	24.33 (29.55)	-60.27	20.25 (26.74)	-61.26	21.20 (27.41)	-60.31	**22.76**
C4 (2.5mM for 24h)	11.88 (20.15)	-81.21	12.34 (20.55)	-79.85	9.26 (17.71)	-82.28	9.03 (17.48)	-83.10	**10.63**
C5 (2.5mM for 36h)	7.30 (15.67)	-88.45	7.66 (16.06)	-87.49	3.52 (10.79)	-93.27	3.93 (11.38)	-92.65	**5.60**
Mean	**28.70**		**28.19**		**23.64**		**23.74**		**26.07**

	SEd	**CD (P=0.05)**
Cultivar	0.183	0.377**
Colchicine	0.224	0.462**
Cultivar X Colchicine	0.448	0.924**

Numbers within parentheses are arc-sine transformed values

Table 13. Effect of oryzalin on the response (%) to multiple shoot regeneration in the first vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	63.22 (52.66)		61.23 (51.48)		52.27 (46.29)		53.42 (46.96)		**57.53**
O1 (10µM for 3d)	56.17 (48.54)	-11.15	51.84 (45.79)	-15.34	44.43 (41.80)	-15.00	45.30 (42.30)	-15.20	**49.43**
O2 (20µM for 3d)	52.25 (46.29)	-17.35	52.42 (46.38)	-14.39	40.40 (39.46)	-22.71	39.31 (38.82)	-26.41	**46.09**
O3 (30µM for 3d)	47.30 (43.45)	-25.18	44.34 (41.74)	-27.59	37.51 (37.76)	-28.23	32.22 (34.58)	-39.69	**40.34**
O4 (40µM for 3d)	41.39 (40.04)	-34.53	41.21 (39.93)	-32.69	30.42 (33.47)	-41.81	29.63 (32.98)	-44.53	**35.66**
O5 (10µM for 6d)	53.73 (47.13)	-15.01	54.34 (47.48)	-11.25	51.00 (45.57)	-2.42	50.50 (45.28)	-5.47	**52.39**
O6 (20µM for 6d)	49.00 (44.42)	-22.49	48.60 (44.19)	-20.63	47.00 (43.28)	-10.07	48.00 (43.85)	-10.15	**48.15**
Mean	**51.86**		**50.56**		**43.29**		**42.63**		**47.09**

	SEd	**CD (P=0.05)**
Cultivar	0.310	0.636**
Oryzalin	0.410	0.841**
Cultivar X Oryzalin	0.821	1.682**

Numbers within parentheses are arc-sine transformed values

Among the cultivars, Sannachenkadali recorded the highest response (51.86%) while Thattillakunnan recorded the lowest (42.63%). Among the treatments, O5 (10µM for 6d) led to the highest response (52.39%), while O4 (40µM for 3d) led to the lowest (35.66%). The values for the interaction effect between cultivars and treatments ranged from 29.63% for the shoot tips of Thattillakunnan treated with O4 (40µM for 3d) to 56.17% for those of Sannachenkadali exposed to O1 (10µM for 3d).

➢ <u>Colchicine Vs oryzalin:</u> The data (Tables 12 and 13) when subjected to 't' test indicated that colchicine and oryzalin varied significantly from each other. Both suppressed the response rates, the degree of suppression being higher with colchicine (an overall mean of 26.07% response to multiple shoot regeneration) than with oryzalin (an overall mean of 47.09% response to multiple shoot regeneration).

The corresponding diploid recorded a higher rate of response (57.53%) than the colchicine and oryzalin treatments.

4.1.1.2.2 Number of microshoots regenerated per culture

(Tables 14 and 15; Fig. 8; Plates 5 and 6)

➢ <u>Effect of colchicine</u>: There existed highly significant variations among the cultivars, the treatments and their interactions (Table 14, Plate 5). Among the cultivars, Anaikomban recorded the highest number of shoots per culture (2.90), while Thattillakunnan recorded the lowest (1.89). Among the treatments, C1 (2.5mM for 12h) regenerated the highest number of shoots per culture (2.73), while C5 (2.5mM for 36h) regenerated the lowest (0.73). The values for the interaction effect ranged between 0.56 shoots per culture for the shoot tips of Thattillakunnan treated with O5 (10µM for 6d) and 3.23 shoots per culture for those of Sannachenkadali treated with O1 (10µM for 3d).

Table 14. Effect of colchicine on the number of microshoots regenerated per culture in the first vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	6.22		6.50		4.40		4.23		**5.33**
C1 (2.5mM for 12h)	3.23	-48.03	3.21	-50.58	2.37	-46.25	2.10	-50.30	**2.73**
C2 (5.0mM for 12h)	2.91	-53.23	2.89	-55.52	2.13	-51.63	1.89	-55.27	**2.45**
C3 (7.5mM for 12h)	2.41	-61.18	2.40	-63.08	1.77	-59.85	1.57	-62.87	**2.04**
C4 (2.5mM for 24h)	1.57	-74.77	1.56	-76.00	1.15	-73.90	1.02	-75.87	**1.32**
C5 (2.5mM for 36h)	0.86	-86.12	0.86	-86.80	0.63	-85.65	0.56	-86.73	**0.73**
Mean	**2.87**		**2.90**		**2.07**		**1.89**		**2.43**

	SEd	CD (P=0.05)
Cultivar	0.034	0.069**
Colchicine	0.041	0.085**
Cultivar X Colchicine	0.082	0.170**

Table 15. *Effect of oryzalin on the number of microshoots regenerated per culture in the first vegetative cycle*

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	6.22		6.50		4.40		4.23		**5.33**
O1 (10µM for 3d)	6.96	11.99	6.91	6.31	7.13	61.93	6.00	42.01	**6.75**
O2 (20µM for 3d)	6.38	2.57	6.00	-7.62	5.97	35.57	5.82	37.63	**6.04**
O3 (30µM for 3d)	4.55	-26.79	5.19	-20.17	5.25	19.20	4.37	3.31	**4.84**
O4 (40µM for 3d)	4.12	-33.79	4.27	-34.33	3.12	-29.09	3.68	-13.02	**3.79**
O5 (10µM for 6d)	4.12	-33.71	4.13	-36.41	3.12	-29.20	3.25	-23.08	**3.65**
O6 (20µM for 6d)	3.19	-48.75	3.55	-45.42	3.47	-21.14	3.67	-13.14	**3.47**
Mean	**5.07**		**5.22**		**4.63**		**4.43**		**4.84**

	SEd	**CD (P=0.05)**
Cultivar	0.199	0.408**
Oryzalin	0.264	0.540**
Cultivar X Oryzalin	0.528	N S

➢ Effect of oryzalin: There existed highly significant variations among the cultivars and the treatments (Table 15, Plate 6). Among the cultivars, Anaikomban regenerated the highest (5.22) and Thattillakunnan the lowest (4.43) number of shoots. Among the treatments, O1 (10μM for 3d) and O2 (20M for 3d) recorded higher number of shoots per culture (6.75 and 6.04 respectively) than that of the corresponding diploid (an average of 5.33 shoots per culture). The lowest number of shoots (3.47) was recorded by the shoot tips subjected to the treatment O6 (20μM for 6d).

➢ Colchicine Vs oryzalin: Subjecting the data (Tables 14 and 15; Fig. 8) to 't' test revealed that colchicine and oryzalin varied significantly from each other. Colchicine (an overall mean of 2.43 shoots per culture) caused a suppression in the multiple shoot regeneration. Oryzalin (an overall mean of 4.84 shoots per culture) on the other hand, had a positive effect on multiple shoot regeneration, with the treatments O1 (10μM for 3d) and O2 (20μM for 3d)) surpassing the corresponding diploid.

The number of shoots regenerated by the corresponding diploid (5.33) was higher than those of the colchicine treatments and lower than those of the oryzalin treatments O1 and O3.

4.1.1.3. Second vegetative cycle

4.1.1.3.1. Response to multiple shoot regeneration (Tables 16 and 17)

➢ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interaction effects (Table 16). Among the cultivars, Anaikomban recorded the highest response (36.49%) and Thattillakunnan the lowest (30.26%). Among the treatments, C1 (2.5mM for 12h) resulted in the highest response (36.27%) and C5 (2.5mM for 36h) the lowest (6.34%). The values for the interaction effect ranged from 3.84% for the shoot tips of Thattillakunnan treated with C5 (2.5mM for 36h) to 42.13% for those of Anaikomban treated with C1 (2.5mM for 12h).

➢ Effect of oryzalin: Treating shoot tips with oryzalin resulted in highly significant variations among the cultivars, the treatments and their interactions (Table 17). Among the cultivars, Sannachenkadali recorded the highest response (69.03%) and Kunnan lowest (65.37%). Among the treatments, O1 (10μM for 3d) resulted in the highest response (71.22%), while O4 (40μM for 3d) resulted in the lowest (54.17%). The treatments involving exposure of shoot tips to lower concentrations for longer duration, O5 (10μM for 6d) and O6 (20μM for 6d) recorded higher rates of response (70.97% and 67.59% respectively) than the treatment involving the highest concentration of oryzalin, O4 (40μM for 3d). The values for the interaction effect between cultivars and oryzalin treatments ranged between 50.40% for the shoot tips of Kunnan treated with O4 (40μM for 3d) and 74.47% for those of Anaikomban treated with O1 (10μM for 3d).

➢ Colchicine Vs oryzalin: 't' test of the data (Tables 16 and 17) revealed that colchicine and oryzalin were statistically different from each other. Both the anti-microtubular compounds suppressed the rate of response to multiple shoot regeneration during the second vegetative cycle. The degree of suppression was higher with colchicine (an overall average of 33.51% response to multiple shoot regeneration) than with oryzalin (an overall average of 67.15% response to multiple shoot regeneration).

The corresponding diploid recorded a higher rate of response (82.13%) than the colchicine and oryzalin treatments.

Table 16. Effect of colchicine on the response (%) to multiple shoot regeneration in the second vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	84.28 (66.63)		82.63 (65.36)		80.33 (63.67)		81.29 (64.36)		**82.13**
C1 (2.5mM for 12h)	37.30 (37.64)	-55.74	42.13 (40.47)	-49.02	33.34 (35.26)	-58.50	32.34 (34.65)	-60.22	**36.27**
C2 (5.0mM for 12h)	33.19 (35.17)	-60.62	35.20 (36.38)	-57.40	30.30 (33.39)	-62.28	26.29 (30.84)	-67.66	**31.24**
C3 (7.5mM for 12h)	30.20 (33.33)	-64.16	30.20 (33.33)	-63.45	26.21 (30.79)	-67.37	21.27 (27.46)	-73.83	**26.97**
C4 (2.5mM for 24h)	20.23 (26.72)	-76.00	21.26 (27.45)	-74.27	14.43 (22.32)	-82.04	16.54 (23.99)	-79.66	**18.11**
C5 (2.5mM for 36h)	9.51 (17.95)	-88.72	7.55 (15.94)	-90.86	4.47 (12.19)	-94.44	3.84 (11.26)	-95.28	**6.34**
Mean	**35.78**		**36.49**		**31.51**		**30.26**		**33.51**

	SEd	CD (P=0.05)
Cultivar	0.122	0.252**
Colchicine	0.150	0.309**
Cultivar X Colchicine	0.300	0.618**

Numbers within parentheses are arc-sine transformed values

Table 17. Effect of oryzalin on the response (%) to multiple shoot regeneration in the second vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	84.28 (66.63)		82.63 (65.36)		80.33 (63.67)		81.29 (64.36)		**82.13**
O1 (10µM for 3d)	73.20 (58.82)	-13.14	74.47 (59.65)	-9.87	69.80 (56.66)	-13.10	67.42 (52.19)	-17.06	**71.22**
O2 (20µM for 3d)	69.40 (56.41)	-17.65	67.26 (55.09)	-18.60	63.55 (52.86)	-20.89	60.16 (50.86)	-25.99	**65.09**
O3 (30µM for 3d)	60.22 (50.89)	-28.54	61.35 (51.55)	-25.75	57.47 (49.29)	-28.45	56.38 (48.66)	-30.65	**58.85**
O4 (40µM for 3d)	54.65 (47.66)	-35.15	53.27 (46.87)	-35.53	50.40 (45.23)	-37.25	58.38 (49.82)	-28.18	**54.17**
O5 (10µM for 6d)	72.21 (58.18)	-14.32	71.63 (57.81)	-13.31	69.83 (56.68)	-13.07	70.21 (56.91)	-13.63	**70.97**
O6 (20µM for 6d)	69.27 (56.33)	-17.80	68.85 (56.07)	-16.68	66.22 (54.46)	-17.56	66.02 (54.34)	-18.79	**67.59**
Mean	**69.03**		**68.49**		**65.37**		**65.69**		**67.15**

	SEd	**CD (P=0.05)**
Cultivar	0.092	0.189**
Oryzalin	0.122	0.250**
Cultivar X Oryzalin	0.244	0.500**

Numbers within parentheses are arc-sine transformed values

4.1.1.3.2. Number of shoots regenerated per culture (Tables 18 and 19; Fig. 8)

- <u>Effect of colchicine:</u> There existed highly significant variations among the cultivars and the treatments (Table 18). Among the cultivars, Sannachenkadali regenerated the highest number of shoots per culture (3.92) and Kunnan the lowest (3.13). Among the treatments, C1 (2.5mM for 12h) resulted in the highest number of shoots per culture (4.50) and C5 (2.5mM for 36h) in the lowest (1.24).
- <u>Effect of oryzalin</u>: Treating shoot tips with oryzalin caused highly significant variations among the cultivars and the treatments (Table 19). Among the cultivars, Sannachenkadali regenerated the highest number of shoots per culture (6.83) and Thattillakunnan the lowest (5.66). Among the treatments, O1 (10μM for 3d) regenerated the highest number of shoots per culture (8.66) which was higher than that of the corresponding diploid (8.27 shoots). The treatment O2 (20μM for 3d) which regenerated 8.25 shoots per culture was on par with the corresponding diploid. The lowest number of shoots (3.34) was regenerated by the treatment O6 (20μM for 6d).
- <u>Colchicine Vs oryzalin:</u> 't' test of the data (Tables 18 and 19; Fig. 8) indicated that colchicine and oryzalin were statistically different from each other. Colchicine caused a higher degree of suppression (an overall average of 3.53), compared to oryzalin (an overall average of 6.17).

The number of shoots regenerated by the corresponding diploid (8.27) was higher than those of the colchicine treatments and lower than those of the oryzalin treatment O1.

Table 18. Effect of colchicine on the number of microshoots regenerated per culture in the second vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	8.87		8.38		7.67		8.18		**8.27**
C1 (2.5mM for 12h)	4.68	-47.26	4.66	-44.39	4.13	-46.12	4.52	-44.77	**4.50**
C2 (5.0mM for 12h)	3.11	-64.92	3.28	-60.92	2.77	-63.93	2.65	-67.65	**2.95**
C3 (7.5mM for 12h)	2.89	-67.40	2.42	-71.18	1.94	-74.76	1.78	-78.29	**2.25**
C4 (2.5mM for 24h)	2.17	-75.58	2.06	-75.42	1.74	-77.36	1.83	-77.68	**1.95**
C5 (2.5mM for 36h)	1.81	-79.64	1.90	-77.33	0.57	-92.56	0.67	-91.87	**1.24**
Mean	**3.92**		**3.78**		**3.13**		**3.27**		**3.53**

	SEd	CD (P=0.05)
Cultivar	0.192	0.396**
Colchicine	0.235	0.485**
Cultivar X Colchicine	0.470	N S

Table 19. Effect of oryzalin on the number of microshoots regenerated per culture in the second vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	8.87		8.38		7.67		8.18		**8.27**
O1 (10μM for 3d)	8.92	0.56	8.95	6.74	8.34	8.81	8.42	3.00	**8.66**
O2 (20μM for 3d)	8.67	-2.20	8.88	5.91	7.69	0.33	7.77	-5.02	**8.25**
O3 (30μM for 3d)	6.19	-30.23	4.88	-41.77	4.68	-39.01	4.57	-44.10	**5.08**
O4 (40μM for 3d)	6.09	-31.30	5.26	-37.29	4.98	-35.09	4.39	-46.30	**5.18**
O5 (10μM for 6d)	5.00	-43.60	5.09	-39.32	3.40	-55.71	4.09	-49.97	**4.39**
O6 (20μM for 6d)	4.12	-53.58	3.94	-52.98	3.11	-59.43	2.20	-73.09	**3.34**
Mean	**6.83**		**6.48**		**5.69**		**5.66**		**6.17**

	SEd	CD (P=0.05)
Cultivar	0.230	0.471**
Oryzalin	0.305	0.624**
Cultivar X Oryzalin	0.609	N S

4.1.1.4. Third vegetative cycle

4.1.1.4.1. Response to multiple shoot regeneration (Tables 20 and 21)

➢ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interactions (Table 20). Among the cultivars, Anaikomban recorded the highest response (38.85%) and Kunnan the lowest (33.71%). Among the treatments, C1 (2.5mM for 12h) resulted in the highest response (38.09%), while C5 (2.5mM for 36h) recorded the lowest (8.15%). Values for the interaction effect ranged between 5.35% for the shoot tips of Kunnan treated with C5 (2.5mM for 36h) and 39.34% for those of Sannachenkadali treated with C1 (2.5mM for 12h).

➢ Effect of oryzalin: Treating shoot tips with oryzalin resulted in highly significant variations among the cultivars, the treatments and their interactions (Table 21). Among the cultivars, Sannachenkadali recorded the highest response (75.95%) and Thattillakunnan the lowest (72.17%). Among the treatments, O1 (10μM for 3d) resulted in the highest response (78.79%), while O4 (40μM for 3d) recorded the lowest (63.22%). The treatments involving exposure of shoot tips to lower concentrations for longer duration, O5 (10M for 6d) and O6 (20M for6d), recorded higher rates of response (76.27% and 69.03% respectively) than the treatment involving the highest concentration, O4 (40M for3d). The values for the interaction effect between cultivars and oryzalin treatments ranged between 60.11% for the shoot tips of Kunnan treated with O4 (40M for3d) and 80.34% for those of Sannachenkadali subjected to O1 (10M for3d).

➢ Colchicine Vs oryzalin: 't' test of the data (Tables 20 and 21) revealed that colchicine and oryzalin were statistically different from each other. The degree of suppression was higher with

Table 20. Effect of colchicine on the response (%) to multiple shoot regeneration in the third vegetative cycle

Treatment	**Sannachenkadali (AA)**		**Anaikomban (AA)**		**Kunnan (AB)**		**Thattillakunnan (AB)**		**Mean**
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	94.19 (76.05)		95.75 (78.27)		89.37 (70.97)		90.20 (71.75)		**92.38**
C1 (2.5mM for 12h)	39.34 (38.84)	-58.24	39.30 (38.81)	-58.96	36.35 (37.07)	-59.33	37.37 (37.68)	-58.57	**38.09**
C2 (5.0mM for 12h)	34.30 (35.84)	-63.59	33.32 (35.25)	-65.20	29.37 (32.81)	-67.14	30.38 (33.44)	-66.32	**31.84**
C3 (7.5mM for 12h)	30.22 (33.34)	-67.91	31.21 (33.96)	-67.40	22.90 (28.57)	-74.38	27.70 (31.75)	-69.29	**28.01**
C4 (2.5mM for 24h)	21.58 (27.68)	-77.09	23.20 (28.79)	-75.77	18.96 (25.80)	-78.79	18.91 (25.76)	-79.04	**20.66**
C5 (2.5mM for 36h)	11.20 (19.54)	-88.11	10.34 (18.75)	-89.21	5.35 (13.36)	-94.02	5.74 (13.84)	-93.64	**8.15**
Mean	**38.47**		**38.85**		**33.71**		**35.05**		**36.52**

	SEd	**CD (P=0.05)**
Cultivar	0.313	0.647**
Colchicine	0.384	0.792**
Cultivar X Colchicine	0.767	1.584**

Numbers within parentheses are arc-sine transformed values

Table 21. Effect of oryzalin on the response (%) to multiple shoot regeneration in the third vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	94.19 (76.05)		95.75 (78.27)		89.37 (70.97)		90.20 (71.75)		**92.38**
O1 (10µM for 3d)	80.34 (63.68)	-14.70	79.47 (63.05)	-17.00	79.18 (62.85)	-11.41	76.19 (60.79)	-15.54	**78.79**
O2 (20µM for 3d)	73.33 (58.90)	-22.15	71.25 (57.57)	-25.59	70.42 (57.05)	-21.20	68.18 (55.66)	-24.41	**70.79**
O3 (30µM for 3d)	70.21 (56.91)	-25.46	72.38 (58.29)	-24.41	67.40 (55.18)	-24.59	64.18 (53.23)	-28.85	**68.54**
O4 (40µM for 3d)	66.13 (54.40)	-29.79	65.26 (53.88)	-31.85	60.11 (50.83)	-32.74	61.37 (51.57)	-31.96	**63.22**
O5 (10µM for 6d)	77.73 (61.84)	-17.48	75.76 (60.50)	-20.87	75.71 (60.47)	-15.28	75.90 (60.60)	-15.85	**76.27**
O6 (20µM for 6d)	69.78 (56.65)	-25.92	68.21 (55.68)	-28.76	68.92 (56.12)	-22.88	69.21 (56.29)	-23.27	**69.03**
Mean	**75.95**		**75.44**		**73.01**		**72.17**		**74.14**

	SEd	CD (P=0.05)
Cultivar	0.261	0.534**
Oryzalin	0.345	0.707**
Cultivar X Oryzalin	0.690	1.414**

Numbers within parentheses are arc-sine transformed values

colchicine (an overall average of 36.52%) than with oryzalin (an overall average of 74.14%).

The corresponding diploid recorded a higher rate of response (92.38%) than the colchicine and oryzalin treatments.

4.1.1.4.2. Number of microshoots regenerated per culture (Tables 22 and 23; Fig. 8)

- <u>Effect of colchicine:</u> The variations among the treatments were highly significant (Table 22). The treatment C1 (2.5mM for 12h) regenerated the highest number of shoots per culture (3.66), while C5 (2.5mM for 36h) regenerated the lowest (1.58).
- <u>Effect of oryzalin:</u> The variations among the cultivars and the treatments were highly significant Table 23). Sannachenkadali regenerated the highest number of shoots per culture (8.95) and Thattillakunnan the lowest (7.40). Among the treatments, O1 (10μM for 3d), resulted in the highest number of shoots per culture (10.35) and O6 (20μM for 6d) the lowest (4.58).
- <u>Colchicine Vs oryzalin:</u> 't' test of the data (Tables 22 and 23; Fig. 8) revealed that the two anti-mitotic agents varied in their effect on the number of microshoots regenerated per culture during the third vegetative cycle. Colchicine caused a higher degree of suppression in the number of microshoots regenerated (an overall average of 4.83 shoots per culture) than oryzalin (an overall average of 8.20 shoots per culture).

The corresponding diploid regenerated higher number of shoots (17.03) than the colchicine and oryzalin treatments.

4.1.1.4.3. Length of microshoots (Tables 24 and 25; Plates 7 and 8)

- <u>Effect of colchicine:</u> There existed highly significant variations among the cultivars and the treatments (Table 24, Plate 7). Among

Table 22. Effect of colchicine on the number of microshoots regenerated per culture in the third vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	17.82		18.79		15.70		15.84		**17.03**
C1 (2.5mM for 12h)	3.65	-79.51	3.84	-79.59	3.39	-78.40	3.77	-76.22	**3.66**
C2 (5.0mM for 12h)	2.78	-84.42	2.76	-85.34	2.81	-82.13	2.27	-85.66	**2.65**
C3 (7.5mM for 12h)	2.70	-84.84	2.17	-88.48	1.88	-88.05	1.84	-88.38	**2.15**
C4 (2.5mM for 24h)	2.37	-86.70	1.64	-91.27	1.79	-88.63	1.78	-88.79	**1.89**
C5 (2.5mM for 36h)	1.91	-89.28	1.83	-90.29	1.34	-91.46	1.24	-92.20	**1.58**
Mean	**5.20**		**5.17**		**4.48**		**4.45**		**4.83**

	SEd	**CD (P=0.05)**
Cultivar	0.427	N S
Colchicine	0.523	1.078**
Cultivar X Colchicine	1.045	N S

Table 23. Effect of oryzalin on the number of microshoots regenerated per culture in the third vegetative cycle

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	No. of shoots	% difference over control	
Control	17.82		18.79		15.70		15.84		**17.03**
O1 (10µM for 3d)	11.37	-36.21	11.71	-37.68	9.16	-41.64	9.16	-42.19	**10.35**
O2 (20µM for 3d)	9.68	-45.66	9.68	-48.51	8.15	-48.10	8.12	-48.72	**8.91**
O3 (30µM for 3d)	6.67	-62.56	5.72	-69.56	5.69	-63.78	5.24	-66.91	**5.83**
O4 (40µM for 3d)	6.19	-65.28	5.25	-72.09	5.33	-66.07	4.78	-69.81	**5.38**
O5 (10µM for 6d)	6.16	-65.45	5.69	-69.72	4.75	-69.74	4.70	-70.32	**5.32**
O6 (20µM for 6d)	4.77	-73.25	5.20	-72.33	4.34	-72.35	4.00	-74.77	**4.58**
Mean	**8.95**		**8.86**		**7.59**		**7.40**		**8.20**

	SEd	CD (P=0.05)
Cultivar	0.442	0.906**
Oryzalin	0.585	1.199**
Cultivar X Oryzalin	1.171	N S

Table 24. Effect of colchicine on the length (cm) of microshoots

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	
Control	9.89		10.46		11.55		11.56		**10.86**
C1 (2.5mM for 12h)	5.23	-47.12	4.21	-59.78	5.87	-49.18	4.74	-58.98	**5.01**
C2 (5.0mM for 12h)	4.28	-56.77	3.78	-63.89	4.64	-59.83	4.51	-60.97	**4.30**
C3 (7.5mM for 12h)	3.91	-60.52	3.46	-66.91	4.58	-60.39	4.52	-60.93	**4.11**
C4 (2.5mM for 24h)	2.69	-72.85	2.52	-75.94	2.75	-76.19	2.69	-76.76	**2.66**
C5 (2.5mM for 36h)	1.60	-83.87	1.74	-83.41	2.82	-75.58	1.66	-85.68	**1.95**
Mean	**4.60**		**4.36**		**5.37**		**4.94**		**4.82**

	SEd	CD (P=0.05)
Cultivar	0.197	0.407**
Colchicine	0.241	0.498**
Cultivar X Colchicine	0.483	N S

Table 25. Effect of oryzalin on the length (cm) of microshoots

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	
Control	9.89		10.46		11.55		11.56		**10.86**
O1 (10µM for 3d)	9.30	-5.97	8.91	-14.83	9.43	-18.35	9.78	-15.36	**9.35**
O2 (20µM for 3d)	8.02	-18.91	7.78	-25.59	8.49	-26.49	8.38	-27.48	**8.17**
O3 (30µM for 3d)	6.19	-37.46	6.21	-40.60	6.36	-44.94	5.77	-50.11	**6.13**
O4 (40µM for 3d)	5.25	-46.97	5.50	-47.39	5.60	-51.52	5.41	-53.18	**5.44**
O5 (10µM for 6d)	6.78	-31.50	6.26	-40.12	6.95	-39.87	6.25	-45.95	**6.56**
O6 (20µM for 6d)	5.95	-39.84	5.23	-49.98	5.44	-52.94	5.50	-52.44	**5.53**
Mean	**7.34**		**7.19**		**7.69**		**7.52**		**7.43**

	SEd	**CD (P=0.05)**
Cultivar	0.186	N S
Oryzalin	0.246	0.505**
Cultivar X Oryzalin	0.493	N S

the cultivars, Kunnan regenerated the longest microshoots (5.37cm) and Anaikomban the shortest (4.36 cm). Among the treatments, C1 (2.5mM for 12h) regenerated the longest microshoots (5.01cm), while C5 (2.5mM for 36h) the shortest (1.95cm).

➢ <u>Effect of oryzalin:</u> Treating shoot tips with oryzalin resulted in significant variations among the treatments (Table 25, Plate 8). Subjecting shoot tips to the treatment O1 (10µM for 3d) resulted in regeneration of the longest microshoots (9.35cm), while O4 (40µM for 3d) resulted in the shortest (5.44cm). The treatments involving exposure of shoot tips to lower concentrations for longer periods, O5 (10µM for 6d) and O6 (20µM for 6d), regenerated longer shoots (6.56cm and 5.53cm respectively) than O4 (40µM for 3d) which involved the highest concentration of oryzalin.

➢ <u>Colchicine Vs oryzalin:</u> The data (Tables 24 and 25) when subjected to 't' test indicated that colchicine and oryzalin varied significantly in their effect on the length of the microshoots. Both the anti-mitotic agents caused a suppression in the length of the microshoots compared to the corresponding diploid, the degree of suppression of microshoot elongation being higher with colchicine (an overall average shoot length of 4.82cm) than with oryzalin (an overall average shoot length of 7.43cm).

The corresponding diploid regenerated longer shoots (10.86cm) than those regenerated by the colchicine and oryzalin treatments.

4.1.1.4.4. Weight of microshoots (Tables 26 and 27)

➢ <u>Effect of colchicine:</u> There existed highly significant variations among the cultivars and the treatments (Table 26). Among the cultivars, microshoots regenerated from Thattillakunnan recorded the highest weight (31.14g), while thosr from kunnan recorded the

Table 26. Effect of colchicine on the weight (g) of microshoots

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	Weight (g)	% difference over control	Weight (g)	% difference over control	Weight (g)	% difference over control	Weight (g)	% difference over control	
Control	68.92		68.38		61.68		63.18		**65.54**
C1 (2.5mM for 12h)	29.65	-56.98	29.20	-57.30	27.25	-55.82	30.47	-51.77	**29.14**
C2 (5.0mM for 12h)	26.49	-61.56	22.45	-67.17	23.21	-62.37	28.33	-55.16	**25.12**
C3 (7.5mM for 12h)	21.01	-69.51	20.25	-70.39	20.66	-66.51	24.04	-61.95	**21.49**
C4 (2.5mM for 24h)	18.82	-72.69	15.45	-77.41	18.20	-70.49	21.16	-66.51	**18.41**
C5 (2.5mM for 36h)	13.82	-79.95	14.02	-79.50	13.71	-77.78	19.69	-68.83	**15.31**
Mean	**29.78**		**28.29**		**27.45**		**31.14**		**29.17**

	SEd	CD (P=0.05)
Cultivar	1.021	2.108**
Colchicine	1.251	2.582**
Cultivar X Colchicine	2.502	N S

Table 27. Effect of oryzalin on the weight (g) of microshoots

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	Weight (g)	% difference over control	Weight (g)	% difference over control	Weight (g)	% difference over control	Weight (g)	% difference over control	
Control	68.92		68.38		61.68		63.18		**65.54**
O1 (10µM for 3d)	43.04	-37.55	41.71	-39.00	48.71	-21.02	46.77	-25.97	**45.06**
O2 (20µM for 3d)	40.68	-40.98	38.95	-43.04	38.68	-37.29	43.70	-30.83	**40.50**
O3 (30µM for 3d)	36.88	-46.48	34.83	-49.06	38.76	-37.15	38.23	-39.49	**37.17**
O4 (40µM for 3d)	27.54	-60.04	23.45	-65.71	22.34	-63.79	23.41	-62.94	**24.18**
O5 (10µM for 6d)	40.89	-40.67	40.08	-41.39	34.78	-43.62	36.80	-41.76	**38.13**
O6 (20µM for 6d)	39.00	-43.42	39.53	-42.20	34.29	-44.40	37.73	-40.28	**37.63**
Mean	**42.42**		**40.99**		**39.89**		**41.40**		**41.17**

	SEd	**CD (P=0.05)**
Cultivar	1.112	N S
Oryzalin	1.470	3.012**
Cultivar X Oryzalin	2.941	N S

lowest weight (27.45g). Among the treatments, C1 (2.5mM for 12h) resulted in the production of microshoots with the highest weight (29.14g), while C5 (2.5mM for 36h) resulted in the lowest (15.31g).

- Effect of oryzalin: Highly significant variations existed among the treatments (Table 27). The treatment O1 (10µM for 3d), resulted in regeneration of microshoots which recorded the highest weight (45.06g), while O4 (40µM for 3d) recorded the lowest weight (24.18g). The treatments involving the lowest concentrations of oryzalin for longer duration, O5 (10µM for 6d) and O6 (20µM for 6d), regenerated microshoots with higher weights (38.13g and 37.63g respectively) than those regenerated from O4 (40µM for 3d).
- Colchicine Vs oryzalin: 't' test of the data (Tables 26 and 27) indicated that colchicine and oryzalin varied significantly in their effect on the weight of the shoots. Both the anti-mitotic agents caused a decline in the weight of the microshoots compared to the corresponding diploid, the degree of decline being higher with colchicine (an overall average shoot weight of 29.17g) than with oryzalin (an overall average shoot weight of 41.17g).

The corresponding diploid regenerated heavier shoots (65.54g) than those regenerated by the colchicine and oryzalin treatments.

4.1.1.4.5. Number of leaves per plantlet (Tables 28 and 29)

- Effect of colchicine: The variations among the cultivars and the treatments were highly significant (Table 28). Among the cultivars, Thattillakunnan recorded the highest number of leaves per plantlet (3.95), while Sannachenkadali recorded the lowest (3.38). Among the treatments, C1 (2.5mM for 12h) resulted in the highest number of leaves per plantlet (4.67), while C5 (2.5mM for 36h) resulted in the lowest (1.91).

Table 28. Effect of colchicine on the number of leaves produced per plantlet

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of leaves	% difference over control	No. of leaves	% difference over control	No. of leaves	% difference over control	No. of leaves	% difference over control	
Control	5.70		5.64		6.28		6.40		**6.00**
C1 (2.5mM for 12h)	4.19	-26.51	4.47	-20.74	4.88	-22.31	5.13	-19.78	**4.67**
C2 (5.0mM for 12h)	3.70	-35.03	3.77	-33.16	4.39	-30.12	4.39	-31.43	**4.06**
C3 (7.5mM for 12h)	2.70	-52.59	3.08	-45.39	3.28	-47.81	3.20	-49.96	**3.06**
C4 (2.5mM for 24h)	2.19	-61.63	2.27	-59.75	2.51	-60.08	2.27	-64.50	**2.31**
C5 (2.5mM for 36h)	1.83	-67.87	1.42	-74.82	2.10	-66.53	2.30	-64.11	**1.91**
Mean	**3.38**		**3.44**		**3.90**		**3.95**		**3.67**

	SEd	CD (P=0.05)
Cultivar	0.143	0.294**
Colchicine	0.175	0.361**
Cultivar X Colchicine	0.349	N S

Table 29. Effect of oryzalin on the number of leaves produced per plantlet

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of leaves	% difference over control	No. of leaves	% difference over control	No. of leaves	% difference over control	No. of leaves	% difference over control	
Control	5.70		5.64		6.28		6.40		**6.00**
O1 (10μM for 3d)	5.63	-1.23	5.80	2.75	5.94	-5.34	5.83	-8.84	**5.80**
O2 (20μM for 3d)	5.02	-11.85	5.17	-8.42	5.34	-14.98	5.27	-17.67	**5.20**
O3 (30μM for 3d)	4.37	-23.35	4.36	-22.78	4.86	-22.55	4.83	-24.47	**4.60**
O4 (40μM for 3d)	4.04	-29.15	4.03	-28.55	4.36	-30.60	4.31	-32.68	**4.18**
O5 (10μM for 6d)	5.08	-10.89	5.27	-6.65	5.69	-9.40	5.49	-14.23	**5.38**
O6 (20μM for 6d)	4.80	-15.80	4.71	-16.49	5.10	-18.73	5.12	-20.02	**4.93**
Mean	**4.94**		**4.99**		**5.36**		**5.32**		**5.16**

	SEd	CD (P=0.05)
Cultivar	0.115	0.236**
Oryzalin	0.152	0.312**
Cultivar X Oryzalin	0.305	N S

➢ Effect of oryzalin: Highly significant variations existed among the cultivars and the treatments (Table 29). Among the cultivars, Kunnan recorded the highest number of leaves per plantlet (5.36) and Sannachenkadali the lowest (4.94). Among the treatments, O1 (10μM for 3d) resulted in the highest number of leaves per plantlet (5.80), while O4 (40μM for 3d) resulted in the lowest (4.18). The treatments involving the lowest concentrations of oryzalin for longer duration, O5 (10μM for 6d) and O6 (20μM for 6d), produced more number of leaves (5.38 and 4.93 respectively) than the treatment O4 (40μM for 3d) which involved the highest concentration of oryzalin.

➢ Colchicine Vs oryzalin: 't' test of the data (Tables 28 and 29) revealed that colchicine and oryzalin varied significantly in their effect on the number of leaves produced per plantlet. Both the anti-mitotic agents caused a decline in the number of leaves produced per plantlet, the decline being higher with colchicine (an overall average of 3.67 leaves per plantlet) than with oryzalin (an overall average of 5.16 leaves per plantlet).

Plantlets of the corresponding diploid had more number of leaves (6.00) than those of the colchicine and oryzalin treatments.

4.1.1.5. *In vitro* rhizogenesis

4.1.1.5.1. Response to rhizogenesis (Tables 30 and 31)

➢ Effect of colchicine: There existed highly significant variations among the cultivars, the treatments and their interactions (Table 30). Among the cultivars, Sannachenkadali recorded the highest response (33.54%) and Kunnan the lowest (29.71%). Among the treatments, C1 (2.5mM for 12h) resulted in the highest response (28.82%) while C5 (2.5mM for 36h) resulted in the lowest (6.48%). Among the interaction effects, the highest response (30.20%) was

Table 30. Effect of colchicine on the response (%) to rhizogenesis

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	95.49 (80.52)		96.97 (80.41)		93.79 (74.03)		91.57 (73.15)		**94.45**
C1 (2.5mM for 12h)	30.20 (33.33)	-68.37	28.36 (32.17)	-70.76	27.39 (31.55)	-70.80	29.32 (32.78)	-67.98	**28.82**
C2 (5.0mM for 12h)	26.29 (30.84)	-72.47	25.41 (30.26)	-73.80	23.30 (28.85)	-75.16	24.18 (29.45)	-73.59	**24.79**
C3 (7.5mM for 12h)	23.36 (28.94)	-75.54	21.38 (27.53)	-77.96	18.31 (25.33)	-80.48	21.36 (27.52)	-76.67	**21.10**
C4 (2.5mM for 24h)	17.33 (24.59)	-81.86	14.50 (22.38)	-85.05	11.26 (19.60)	-88.00	11.15 (19.50)	-87.83	**13.56**
C5 (2.5mM for 36h)	8.60 (17.04)	-91.00	7.73 (16.13)	-92.03	4.22 (11.85)	-95.50	5.38 (13.40)	-94.13	**6.48**
Mean	**33.54**		**32.39**		**29.71**		**30.49**		**31.53**

	SEd	CD (P=0.05)
Cultivar	0.386	0.796**
Colchicine	0.473	0.975**
Cultivar X Colchicine	0.945	1.951**

Numbers within parentheses are arc-sine transformed values

Table 31. Effect of oryzalin on the response (%) to rhizogenesis

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% response	% difference over control	% response	% difference over control	% response	% difference over control	% response	% difference over control	
Control	95.49 (80.52)		96.97 (80.41)		93.79 (74.03)		91.57 (73.15)		**94.45**
O1 (10µM for 3d)	67.21 (55.06)	-29.61	69.39 (56.41)	-28.44	62.47 (52.22)	-33.39	63.20 (52.65)	-30.98	**65.57**
O2 (20µM for 3d)	63.44 (52.79)	-33.57	65.18 (53.83)	-32.79	61.30 (51.53)	-34.64	61.35 (51.56)	-33.00	**62.82**
O3 (30µM for 3d)	60.34 (50.96)	-36.81	59.29 (50.35)	-38.86	55.82 (48.34)	-40.48	57.27 (49.18)	-37.46	**58.18**
O4 (40µM for 3d)	59.22 (50.31)	-37.98	57.31 (49.20)	-40.90	54.21 (47.41)	-42.20	53.80 (47.18)	-41.25	**56.14**
O5 (10µM for 6d)	65.33 (53.92)	-31.59	64.40 (53.36)	-33.59	58.19 (49.71)	-37.95	59.22 (50.31)	-35.33	**61.78**
O6 (20µM for 6d)	60.30 (50.94)	-36.85	58.29 (49.77)	-39.89	54.17 (47.39)	-42.24	56.21 (48.56)	-38.62	**57.24**
Mean	**67.33**		**67.26**		**62.85**		**63.23**		**65.17**

	SEd	**CD (P=0.05)**
Cultivar	0.465	0.953**
Oryzalin	0.615	1.260**
Cultivar X Oryzalin	1.230	N S

Numbers within parentheses are arc-sine transformed values

recorded by the shoot tips of Sannachenkadali treated with C1 (2.5mM for 12h) and the lowest (4.22%) was recorded by those of Kunnan treated with C5 (2.5mM for 36h).

➢ <u>Effect of oryzalin:</u> Highly significant variations existed among the cultivars and the treatments (Table 31). Among the cultivars, Sannachenkadali recorded the highest response (67.33%) and Kunnan the lowest (62.85%). Among the treatments, O1 (10μM for 3d) resulted in the highest response (65.57%), while O4 (40μM for 3d) resulted in the lowest (56.14%). The treatments involving exposure of shoot tips to lower concentrations for longer periods, O5 (10μM for 6d) and O6 (20μM for 6d), recorded higher response (61.78% and 57.24% respectively) than O4 (40μM for 3d) which involved the highest concentration of oryzalin.

➢ <u>Colchicine Vs oryzalin:</u> 't' test of the data (Tables 30 and 31) indicated that colchicine and oryzalin were statistically different from each other with respect to the degree of suppression caused on the rate of response to *in vitro* rhizogenesis. The degree of suppression was higher with colchicine (an overall average response of 31.53%) than with oryzalin (an overall average response of 65.17%).

The corresponding diploid recorded a higher rate of response (94.45%) than the colchicine and oryzalin treatments.

4.1.1.5.2. Time taken for root initiation (Tables 32 and 33)

➢ <u>Effect of colchicine:</u> Highly significant variations existed among the cultivars, the treatments and their interactions (Table 32). Among the cultivars, Anaikomban recorded the earliest root initiation (33.56d after transfer to rooting medium) and Thattillakunnan recorded the most delayed root initiation (36.53d). Among the treatments, C1 (2.5mM for 12h) resulted in the earliest root initiation (33.41d) and C5 (2.5mM for 36h) resulted in the

Table 32. Effect of colchicine on the time taken (days after transfer to rooting medium) for root initiation

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	14.85		14.29		17.32		18.47		**16.23**
C1 (2.5mM for 12h)	31.30	110.85	31.86	123.03	34.79	100.90	35.71	93.31	**33.41**
C2 (5.0mM for 12h)	33.69	126.91	31.68	121.77	36.41	110.28	33.15	79.48	**33.73**
C3 (7.5mM for 12h)	36.79	147.79	37.25	160.73	39.70	129.28	41.23	123.20	**38.74**
C4 (2.5mM for 24h)	42.25	184.57	42.02	194.12	42.82	147.27	44.26	139.60	**42.83**
C5 (2.5mM for 36h)	43.22	191.14	44.28	209.94	45.30	161.59	46.35	150.92	**44.78**
Mean	**33.68**		**33.56**		**36.05**		**36.53**		**34.95**

	SEd	CD (P=0.05)
Cultivar	0.228	0.471**
Colchicine	0.279	0.577**
Cultivar X Colchicine	0.559	1.153**

Table 33. Effect of oryzalin on the time taken (days after transfer to rooting medium) for root initiation

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	14.85		14.29		17.32		18.47		**16.23**
O1 (10μM for 3d)	21.20	42.81	20.68	44.73	22.67	30.90	23.31	26.18	**21.96**
O2 (20μM for 3d)	24.32	63.83	23.91	67.38	25.23	45.71	25.88	40.09	**24.83**
O3 (30μM for 3d)	28.23	90.13	27.90	95.27	29.86	72.45	29.71	60.83	**28.92**
O4 (40μM for 3d)	31.19	110.07	30.77	115.37	31.27	80.59	30.80	66.76	**31.01**
O5 (10μM for 6d)	23.72	59.78	22.91	60.34	24.18	39.62	25.27	36.79	**24.02**
O6 (20μM for 6d)	25.70	73.12	25.75	80.22	26.73	54.35	27.21	47.32	**26.35**
Mean	**24.17**		**23.74**		**25.32**		**25.80**		**24.76**

	SEd	CD (P=0.05)
Cultivar	0.309	0.632**
Oryzalin	0.408	0.836**
Cultivar X Oryzalin	0.816	N S

most delayed root initiation (44.78d). Among the interaction effects, the earliest root initiation (31.30d) was recorded by the cultures of Sannachenkadali treated with C1 (2.5mM for 12h), while the most delayed root initiation (46.35d) was recorded by those of Thattillakunnan treated with C5 (2.5mM for 36h).

➢ <u>Effect of oryzalin:</u> Highly significant variations existed among the cultivars and the treatments (Table 33). Among the cultivars, Anaikomban was found to require the least duration (23.74d) for root initiation, whereas cultures of Thattillakunnan took the longest time (25.80d) to initiate roots. Among the treatments, O1 (10μM for 3d) resulted in the earliest root initiation (21.96d), while O4 (40μM for 3d) resulted in the most delayed response (31.01d). The treatments involving exposure of shoot tips to lower concentrations for longer periods, O5 (10μM for 6d) and O6 (20μM for 6d) took comparatively lesser time (24.02d and 26.35d respectively) than the treatmment involving the highest concentration of oryzalin, O4 (40μM for 3d) to initiate roots.

➢ <u>Colchicine Vs oryzalin:</u> The data (Tables 32 and 33) when subjected to 't' test revealed that the anti-mitoticagents varied in their effect on the time taken for initiation of roots. Both delayed the process of root initiation, the delay being greater with the colchicine treatments (an overall average of 34.95d for root initiation) than with the oryzalin treatments (an overall average of 24.76d).

The corresponding diploid took lesser time (16.23d) than the colchicine and oryzalin treatments to initiate roots.

4.1.1.5.3. Number of roots per plantlet (Tables 34 and 35; Plates 9 and 10)

➢ <u>Effect of colchicine:</u> Highly significant variations existed among the cultivars and the treatments (Table 34, Plate 9). Among the

Table 34. Effect of colchicine on the number of roots produced per plantlet

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of roots	% difference over control	No. of roots	% difference over control	No. of roots	% difference over control	No. of roots	% difference over control	
Control	9.79		9.75		8.67		7.99		**9.05**
C1 (2.5mM for 12h)	4.34	-55.70	4.27	-56.18	4.21	-51.47	4.00	-49.91	**4.20**
C2 (5.0mM for 12h)	3.12	-68.11	3.05	-68.75	2.00	-76.92	2.00	-74.95	**2.54**
C3 (7.5mM for 12h)	1.75	-82.12	2.10	-78.45	1.57	-81.94	1.64	-79.52	**1.76**
C4 (2.5mM for 24h)	0.99	-89.88	1.30	-86.66	1.15	-86.73	0.80	-89.98	**1.06**
C5 (2.5mM for 36h)	0.07	-99.34	0.60	-93.84	0.50	-94.23	0.45	-94.36	**0.40**
Mean	**3.34**		**3.51**		**3.01**		**2.81**		**3.17**

	SEd	**CD (P=0.05)**
Cultivar	0.244	0.503**
Colchicine	0.299	0.616**
Cultivar X Colchicine	0.597	N S

Table 35. Effect of oryzalin on the number of roots produced per plantlet

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	No. of roots	% difference over control	No. of roots	% difference over control	No. of roots	% difference over control	No. of roots	% difference over control	
Control	9.79		9.75		8.67		7.99		**9.05**
O1 (10μM for 3d)	8.50	-13.18	8.27	-15.19	7.02	-19.04	7.32	-8.39	**7.77**
O2 (20μM for 3d)	6.66	-31.99	6.72	-31.04	5.85	-32.54	5.34	-33.12	**6.14**
O3 (30μM for 3d)	5.57	-43.08	4.92	-49.51	4.37	-49.62	3.41	-57.29	**4.57**
O4 (40μM for 3d)	4.61	-52.94	4.59	-52.95	3.91	-54.88	3.45	-56.79	**4.14**
O5 (10μM for 6d)	6.30	-35.67	6.07	-37.71	5.78	-33.35	5.25	-34.25	**5.85**
O6 (20μM for 6d)	5.11	-47.78	4.92	-49.51	5.07	-41.49	3.91	-51.10	**4.75**
Mean	**6.65**		**6.46**		**5.81**		**5.24**		**6.04**

	SEd	CD (P=0.05)
Cultivar	0.220	0.451**
Oryzalin	0.291	0.596**
Cultivar X Oryzalin	0.582	N S

cultivars, Anaikomban recorded the highest number of roots per plantlet (3.51), while Thattillakunnan recorded the lowest (2.81). Among the treatments, C1 (2.5mM for 12h) resulted in the highest number of roots per plantlet (4.20), while C5 (2.5mM for 36h) resulted in the lowest number (0.40).

- Effect of oryzalin: Highly significant variations were observed among the cultivars and the treatments (Table 35, Plate 10). The cultivar Sannachenkadali recorded the highest number of roots per plantlet (6.65) and Thattillakunnan the lowest (5.24). Among the treatments O1 (10µM for 3d) resulted in production of the highest number of roots per plantlet (7.77), while O4 (40µM for 3d) resulted in the lowest (4.14). The treatments involving exposure of shoot tips to lower concentrations for longer periods, O5 (10µM for 6d) and O6 (20µM for 6d), produced higher number of roots per plantlet (5.85 and 4.75 respectively) than O4 (40µM for 3d) which involved the highest concentration of oryzalin.
- Colchicine Vs oryzalin: Subjecting the data (Tables 34 and 35) to 't' test indicated that colchicine and oryzalin varied significantly in their effect on the number of roots produced per plantlet. Both the anti-mitotic agents suppressed the root production, the degree of suppression caused being higher with colchicine (an overall average of 3.17 roots per plantlet) than with oryzalin (an overall average of 6.04 roots per plantlet).

The corresponding diploid produced lower number of roots (9.05) than the colchicine and oryzalin treatments.

4.1.1.5.4. Length of roots (Tables 36 and 37, Plates 11 and 12)

- Effect of colchicine: Highly significant variations existed among the treatments (Table 36, Plate 11). The longest roots (4.30cm) were produced by the treatment C1 (2.5mM for 12h), while the shortest (0.89cm) were produced by C5 (2.5mM for 36h).

Table 36. Effect of colchicine on the length (cm) of roots

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	
Control	13.26		12.68		12.71		11.19		**12.46**
C1 (2.5mM for 12h)	4.92	-62.90	4.67	-63.16	3.80	-70.13	3.81	-65.94	**4.30**
C2 (5.0mM for 12h)	3.62	-72.70	3.74	-70.53	4.16	-67.30	3.42	-69.42	**3.73**
C3 (7.5mM for 12h)	3.32	-75.00	3.78	-70.22	2.93	-76.94	3.18	-71.57	**3.30**
C4 (2.5mM for 24h)	2.22	-83.30	2.48	-80.47	2.02	-84.10	2.30	-79.48	**2.25**
C5 (2.5mM for 36h)	0.89	-93.29	1.04	-91.79	0.85	-93.35	0.80	-92.89	**0.89**
Mean	**4.70**		**4.73**		**4.41**		**4.11**		**4.49**

	SEd	CD (P=0.05)
Cultivar	0.310	N S
Colchicine	0.380	0.784**
Cultivar X Colchicine	0.760	N S

Table 37. Effect of oryzalin on the length (cm) of roots

Treatment	**Sannachenkadali (AA)**		**Anaikomban (AA)**		**Kunnan (AB)**		**Thattillakunnan (AB)**		**Mean**
	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	Length (cm)	% difference over control	
Control	13.26		12.68		12.71		11.19		**12.46**
O1 (10µM for 3d)	7.64	-42.38	8.44	-33.41	6.28	-50.57	7.50	-32.95	**7.47**
O2 (20µM for 3d)	6.29	-52.60	6.49	-48.80	5.06	-60.17	4.50	-59.77	**5.58**
O3 (30µM for 3d)	4.73	-64.33	5.28	-58.34	4.78	-62.38	3.50	-68.71	**4.57**
O4 (40µM for 3d)	3.91	-70.51	3.58	-71.79	2.50	-80.32	3.00	-73.18	**3.25**
O5 (10µM for 6d)	6.61	-50.15	6.07	-52.15	5.67	-55.41	5.72	-48.86	**6.02**
O6 (20µM for 6d)	4.00	-69.83	3.50	-72.39	3.50	-72.45	3.50	-68.71	**3.63**
Mean	**6.63**		**6.58**		**5.78**		**5.56**		6.14

	SEd	**CD (P=0.05)**
Cultivar	0.316	0.648**
Oryzalin	0.418	0.857**
Cultivar X Oryzalin	0.837	N S

- Effect of oryzalin: Highly significant variations existed among the cultivars and the treatments (Table 37, Plate 12). The cultivar Sannachenkadali produced the longest roots (6.63cm), whereas Thattillakunnan produced the shortest (5.56cm). Among the treatments, O1 (10μM for 3d) produced the longest roots (7.47cm), while O4 (40μM for 3d) resulted in the shortest roots (3.25cm).
- Colchicine Vs oryzalin: The data (Tables 36 and 37) when subjected to 't' test revealed that the two anti-mitotic agents varied significantly in their effect on the length of the roots. Both suppressed the root elongation, the degree of suppression caused being higher with colchicine (an overall average root length of 4.49cm) than with oryzalin (an overall average root length of 6.14cm). The treatments involving exposure of shoot tips to lower concentrations for longer periods, O5 (10μM for 6d) and O6 (20μM for 6d), produced longer roots (6.02cm and 3.63cm respectively) than O4 (40μM for 3d) which involved the highest concentration of oryzalin.

The corresponding diploid produced longer roots (12.46cm) than the colchicine and oryzalin treatments.

4.1.1.6. Total *in vitro* duration (Tables 38 and 39)

- Effect of colchicine: There existed highly significant variations among the cultivars and the treatments (Table 38). Among the cultivars, Sannachenkadali recorded the shortest *in vitro* phase (166.91d) and Thattillakunnan underwent the longest *in vitro* phase (171.98d). Among the treatments, C1 (2.5mM for 12h) resulted in the shortest *in vitro* phase (157.38d), whereas C5 (2.5mM for 36h) resulted in the longest *in vitro* phase (200.26d).
- Effect of oryzalin: Highly significant variations existed among the cultivars and the treatments (Table 39). Among the cultivars, Anaikomban was found to require the lowest number of days

Table 38. **Effect of colchicine on the total *in vitro* duration (days)**

Treatment	**Sannachenkadali (AA)**		**Anaikomban (AA)**		**Kunnan (AB)**		**Thattillakunnan (AB)**		**Mean**
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	128.05		128.51		132.58		134.36		**130.87**
C1 (2.5mM for 12h)	154.55	20.70	154.67	20.36	159.97	20.66	160.32	19.33	**157.38**
C2 (5.0mM for 12h)	163.39	27.59	161.56	25.72	168.55	27.13	165.77	23.38	**164.82**
C3 (7.5mM for 12h)	172.26	34.53	173.32	34.86	176.10	32.82	177.61	32.19	**174.82**
C4 (2.5mM for 24h)	185.53	44.88	185.95	44.69	188.00	41.80	190.66	41.91	**187.53**
C5 (2.5mM for 36h)	197.67	54.37	199.00	54.85	201.22	51.77	203.17	51.22	**200.26**
Mean	**166.91**		**167.17**		**171.07**		**171.98**		**169.28**

	SEd	**CD (P=0.05)**
Cultivar	0.420	0.867**
Colchicine	0.515	1.062**
Cultivar X Colchicine	1.029	N S

Table 39. **Effect of oryzalin on the total *in vitro* duration (days)**

Treatment	**Sannachenkadali (AA)**		**Anaikomban (AA)**		**Kunnan (AB)**		**Thattillakunnan (AB)**		**Mean**
	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	No. of days	% difference over control	
Control	128.05		128.51		132.58		134.36		**130.87**
O1 (10µM for 3d)	137.02	7.01	136.88	6.51	139.65	5.33	139.23	3.62	**138.19**
O2 (20µM for 3d)	144.58	12.91	143.69	11.81	145.92	10.06	145.61	8.37	**144.95**
O3 (30µM for 3d)	152.90	19.40	153.40	19.36	155.74	17.47	155.77	15.94	**154.45**
O4 (40µM for 3d)	161.08	25.79	161.10	25.36	162.02	22.20	162.74	21.13	**161.73**
O5 (10µM for 6d)	142.29	11.12	140.67	9.46	143.98	8.59	145.69	8.43	**143.15**
O6 (20µM for 6d)	148.89	16.27	148.71	15.72	150.47	13.49	151.99	13.13	**150.01**
Mean	**144.97**		**144.71**		**147.19**		**147.91**		**146.19**

	SEd	**CD (P=0.05)**
Cultivar	0.343	0.703**
Oryzalin	0.454	0.930**
Cultivar X Oryzalin	0.908	N S

(144.71) to complete the *in vitro* phase and Thattillakunnan the highest number of days (147.91). Among the treatments, O1 (10µM for 3d) resulted in the shortest (138.19d) *in vitro* phase, while O4 (40µM for 3d) resulted in the most extended *in vitro* phase (161.73d). The treatments involving the lowest concentrations for longer duration, namely O5 (10µM for 6d) and O6 (20µM for 6d) recorded *in vitro* phases shorter (143.15 days and 150.01 days respectively) than that of O4 (40µM for 3d).

➢ Colchicine Vs oryzalin: 't' test of the data (Tables 38 and 39) revealed that colchicine and oryzalin varied significantly in their effect on the time required for the *in vitro* cultures to complete their *in vitro* phase. Both the anti-mitotic agents extended the *in vitro* phase of the cultures and the degree of extension was higher with colchicine (an overall average *in vitro* duration of 169.28d) than with oryzalin (an overall average *in vitro* duration of 146.19d).

The total *in vitro* duration of the corresponding diploid was shorter (130.87d) than those of the colchicine and oryzalin treatments.

4.1.1.7. ***Ex vitro* survival rates of *in vitro* derived plantlets**

4.1.1.7.1. **Two weeks after transfer to *ex vitro* environment**

(Tables 40 and 41)

➢ Effect of colchicine: The variations among the treatments were highly significant (Table 40). The treatment C1 (2.5mM for 12h) resulted in the highest survival rate (41.42%) and C5 (2.5mM for 36h) the lowest (8.99%).

➢ Effect of oryzalin: The variations among the treatments were highly significant (Table 41). The treatment O1 (10µM for 3d) recorded the highest survival rate (73.20%) and the treatment O6 (20µM for 6d) the lowest (68.64%). The treatment involving the lowest concentration of oryzalin for longer duration, O5 (10µM for

Table 40. *Ex vitro* **survival rates (%) of regenerants derived from colchicine-treated cultures two weeks after transfer to *ex vitro* environment**

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% survival	% difference over control	% survival	% difference over control	% survival	% difference over control	No. of survival	% difference over control	
Control	93.54 (75.27)		94.43 (76.34)		94.29 (76.17)		92.92 (74.58)		**93.79**
C1 (2.5mM for 12h)	41.61 (40.17)	-55.52	40.63 (39.59)	-56.98	41.24 (39.95)	-56.26	42.20 (40.51)	-54.58	**41.42**
C2 (5.0mM for 12h)	37.23 (37.60)	-60.20	36.18 (36.97)	-61.69	35.67 (36.66)	-62.17	35.19 (36.37)	-62.13	**36.07**
C3 (7.5mM for 12h)	30.18 (33.32)	-67.74	30.15 (33.30)	-68.07	28.76 (32.43)	-69.50	28.83 (32.46)	-68.98	**29.48**
C4 (2.5mM for 24h)	20.16 (26.68)	-78.45	20.68 (27.04)	-78.10	19.73 (26.36)	-79.08	20.32 (26.78)	-78.13	**20.22**
C5 (2.5mM for 36h)	9.17 (17.62)	-90.20	8.75 (17.20)	-90.73	8.85 (17.30)	-90.61	9.20 (17.65)	-90.10	**8.99**
Mean	**38.65**		**38.47**		**38.09**		**38.11**		**38.33**

	SEd	**CD (P=0.05)**	
Cultivar	0.312	N S	N S
Colchicine	0.382	0.788**	H.S
Cultivar X Colchicine	0.764	N S	N S

Numbers within parentheses are arc-sine transformed values

Table 41. ***Ex vitro*** **survival rates (%) of regenerants derived from oryzalin-treated cultures two weeks after transfer to *ex vitro* environment**

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% survival	% difference over control	% survival	% difference over control	% survival	% difference over control	No. of survival	% difference over control	
Control	93.54 (75.27)		94.43 (76.34)		94.29 (76.17)		92.92 (74.58)		**93.79**
O1 (10µM for 3d)	74.35 (59.58)	-20.52	75.00 (60.32)	-20.57	71.58 (58.03)	-24.09	71.89 (57.99)	-22.63	**73.20**
O2 (20µM for 3d)	73.24 (58.84)	-21.71	71.37 (57.65)	-24.42	69.31 (56.36)	-26.49	71.75 (57.89)	-22.78	**71.41**
O3 (30µM for 3d)	71.29 (57.59)	-23.79	69.89 (56.71)	-25.99	65.77 (54.19)	-30.24	68.68 (55.96)	-26.09	**68.90**
O4 (40µM for 3d)	70.92 (57.36)	-24.19	69.77 (56.64)	-26.12	69.25 (56.32)	-26.55	69.64 (56.56)	-25.05	**69.89**
O5 (10µM for 6d)	73.20 (58.82)	-21.75	72.75 (58.53)	-22.96	68.78 (56.23)	-27.06	69.35 (56.39)	-25.36	**71.02**
O6 (20µM for 6d)	69.73 (56.61)	-25.46	68.86 (56.08)	-27.07	66.67 (54.73)	-29.29	69.32 (56.36)	-25.40	**68.64**
Mean	**75.18**		**74.58**		**72.23**		**73.36**		**73.84**

	SEd	**CD (P=0.05)**
Cultivar	1.000	N S
Oryzalin	1.323	2.710**
Cultivar X Oryzalin	2.646	N S

Numbers within parentheses are arc-sine transformed values

6d) recorded a higher survival rate (71.02%) than that of O4 (40µM for 3d) which recorded 69.89% survival.

- Colchicine Vs oryzalin: 't' test of the data (Tables 40 and 41) revealed that colchicine and oryzalin varied significantly from each other. Both the anti-mitotic agents were associated with a decline in the *ex vitro* survival rates, the decline being higher with colchicine (an overall average *ex vitro* survival rate of 38.33%) than for oryzalin (an overall average *ex vitro* survival of 73.84%).

The corresponding diploid recorded a higher *ex vitro* survival rate (93.79%) than the colchicine and oryzalin treatments, two weeks after transfer to *ex vitro* environment.

4.1.1.7.2. Two months after transfer to *ex vitro* environment

(Tables 42 and 43)

- Effect of colchicine: There existed highly significant variations among the cultivars and the treatments (Table 42). Among the cultivars, Anaikomban recorded the highest survival rate (31.40%), while Thattillakunnan recorded the lowest (29.09%). Among the treatments, C1 (2.5mM for 12h) resulted in the highest survival rate (29.76%) and the treatment C5 (2.5mM for 36h) resulted in the lowest (1.57%).
- Effect of oryzalin: The variations among the cultivars were significant and those among the treatments were highly significant (Table 43). Among the cultivars, Sannachenkadali recorded the highest survival rate (69.03%) and Thattillakunnan recorded the lowest (63.99%). Among the treatments, the highest survival rate of 67.84% was recorded by the treatment O1 (10µM for 3d), while the lowest of 56.78% was recorded by the treatment O4 (40µM for 3d). The treatments involving the lowest concentrations of oryzalin for longer duration, O5 (10µM for 6d) and O6 (20µM for 6d) recorded

Table 42. ***Ex vitro*** **survival rates (%) of regenerants derived from colchicine-treated cultures two months after transfer to** ***ex vitro*** **environment**

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% survival	% difference over control	% survival	% difference over control	% survival	% difference over control	No. of survival	% difference over control	
Control	91.69 (73.25)		91.73 (73.29)		92.21 (73.79)		90.24 (71.79)		**91.46**
C1 (2.5mM for 12h)	31.82 (34.33)	-65.30	30.78 (33.69)	-66.44	29.79 (33.07)	-67.69	26.67 (31.07)	-70.44	**29.76**
C2 (5.0mM for 12h)	26.23 (30.80)	-71.39	26.69 (31.10)	-70.91	26.17 (30.76)	-71.62	24.77 (29.84)	-72.55	**25.96**
C3 (7.5mM for 12h)	24.12 (29.41)	-73.70	25.27 (30.16)	-72.45	24.29 (29.52)	-73.66	22.82 (28.53)	-74.72	**24.12**
C4 (2.5mM for 24h)	11.23 (19.56)	-87.76	12.19 (20.41)	-86.71	10.26 (18.67)	-88.88	9.15 (17.58)	-89.86	**10.71**
C5 (2.5mM for 36h)	2.28 (8.68)	-97.51	1.75 (7.52)	-98.10	1.35 (6.65)	-98.54	0.90 (5.28)	-99.01	**1.57**
Mean	**31.23**		**31.40**		**30.67**		**29.09**		**30.60**

	SEd	CD (P=0.05)
Cultivar	0.411	0.847**
Colchicine	0.503	1.038**
Cultivar X Colchicine	1.006	N S

Numbers within parentheses are arc-sine transformed values

Table 43. *Ex vitro* **survival rates (%) of regenerants derived from oryzalin-treated cultures two months after transfer to *ex vitro* environment**

Treatment	Sannachenkadali (AA)		Anaikomban (AA)		Kunnan (AB)		Thattillakunnan (AB)		Mean
	% survival	% difference over control	% survival	% difference over control	% survival	% difference over control	No. of survival	% difference over control	
Control	91.69 (73.25)		91.73 (73.29)		92.21 (73.79)		90.24 (71.79)		**91.46**
O1 (10µM for 3d)	70.87 (57.33)	-22.71	69.21 (56.43)	-24.55	66.62 (54.77)	-27.75	64.68 (53.53)	-28.33	**67.84**
O2 (20µM for 3d)	67.83 (55.45)	-26.02	67.14 (55.02)	-26.80	66.42 (54.58)	-27.97	61.77 (51.81)	-31.55	**65.79**
O3 (30µM for 3d)	61.21 (51.47)	-33.24	60.92 (51.30)	-33.59	64.83 (51.84)	-29.69	56.69 (48.84)	-37.18	**60.91**
O4 (40µM for 3d)	59.10 (50.24)	-35.54	56.97 (49.00)	-37.90	55.35 (48.07)	-39.97	55.69 (48.26)	-38.29	**56.78**
O5 (10µM for 6d)	67.81 (55.43)	-26.04	66.24 (54.61)	-27.79	62.22 (52.13)	-32.52	59.66 (50.57)	-33.88	**63.98**
O6 (20µM for 6d)	64.75 (53.57)	-29.38	61.22 (51.48)	-33.26	63.32 (52.72)	-31.33	59.19 (50.29)	-34.40	**62.12**
Mean	**69.03**		**67.63**		**67.28**		**63.99**		**66.98**

	SEd	CD (P=0.05)
Cultivar	0.872	1.786*
Oryzalin	1.154	2.363**
Cultivar X Oryzalin	2.307	N S

Numbers within parentheses are arc-sine transformed values

higher survival rates (63.98% and 62.12% respectively) than that of O4 (40μM for 3d).

- Colchicine Vs oryzalin: 't' test of the data (Tables 42 and 43) revealed that colchicine and oryzalin varied significantly from each other. Both the anti-mitotic agents were associated with a decline in the *ex vitro* survival rates, the decline being higher with colchicine (an overall average *ex vitro* survival of 30.60%) than with oryzalin (an overall average *ex vitro* survival of 66.98%).

The corresponding diploid recorded a higher *ex vitro* survival rate (91.46%) than the colchicine and oryzalin treatments, two months after transfer to *ex vitro* environment.

4.2 PLOIDY ASSESSMENT OF *IN VITRO* REGENERATED PLANTS

4.2.1. Preliminary ploidy-screening

4.2.1.1. Stomata analyses (Tables 44, 45, 46; Plates 14 to 22, 26)

Based on microscopic measurement of the density and size of stomata in the leaves of *in vitro* derived plants, the plants were initially ploidy-screened. Plants with stomatal densities ranging between 39.00 and 50.00 stomata mm^{-2} and stomata size (Length x Breadth μ^2) between 950 and 1200μ^2 were categorised as diploids (Plates 16, 17, 21) and those with stomatal densities between 12.00 and 19.00 stomata mm^{-2} and stomatal dimensions between 1850 and 2250μ^2 were categorised as tetraploids (Plates 14, 15, 19, 20). Those with stomatal densities below 8.00 stomata mm^{-2} and stomatal dimensions above 2500μ^2 were categorised as octoploids (Plates 18, 22). Plants which had mixtures of two or more of these stomatal densities and dimensions were categorised as mixoploids (Plate 26). Since the frequency of occurrence of octoploid stomata was lower, the octoploids and mixoploids were collectively categorised as 'others'.

The stomatal traits of the *in vitro* derived plantlets are presented in Table 44.

- **Plants regenerated from colchicine-treated cultures**

The data (Table 45) revealed that none of the plants regenerated from the cultures treated with C1 (2.5mM for 12h) had stomatal densities and dimensions characteristic of tetraploids. The percentages of plants with stomatal characters of 'other' ploids were 14.29, 8.33 and 22.22 respectively for the cultivars Sannachenkadali, Anaikomban and Kunnan respectively.

The percentages of plants with tetraploid stomatal characters among those regenerated from cultures treated with C2 (5.0mM for

Table 44. Stomata and chloroplast traits of *in vitro* regenerated plantlets

Cultivar	Ploidy level	Stomatal density (No. of stomata mm^{-2})		Stomata size (Length x Breadth μ^2)		Chloroplast density No. of chloroplasts GCP^{-1}	
		Mean	Range	Mean	Range	Mean	Range
Sannachenkadali	2n	42.92	39.16 - 53.24	1085.26	1004 - 1132	10.00	9.00 - 11.00
	4n	16.28	14.23 - 18.31	2126.33	2055 - 2210	16.00	15.00 - 17.00
Anaikomban	2n	49.32	46.24 - 54.23	1012.41	988 - 1073	10.00	9.00 - 11.00
	4n	15.19	13.16 - 17.21	2025.52	1850 - 2158	16.00	15.00 - 17.00
Kunnan	2n	50.16	47.32 - 52.61	1052.14	950 - 1116	10.00	9.00 - 11.00
	4n	14.28	13.18 - 16.14	2036.18	2005 - 2165	16.00	15.00 - 17.00
Thattillakunnan	2n	50.26	47.32 - 54.24	1126.58	1053 - 1200	10.00	9.00 - 11.00
	4n	17.14	14.29 - 19.32	2185.09	1955 - 2250	16.00	15.00 - 17.00
All cultivars	8n	5.91	5.04 - 7.46	2673.14	2512 - 2738	20.00	19.00 - 21.00

Table 45. Ploidy status (% of different ploidy levels) of regenerants derived from colchicine-treated cultures as assessed by stomata analyses

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			Mean		
	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***
C1 (2.5mM for 12h)	-	85.71	14.29	-	91.67	8.33	-	77.78	22.22	-	100.00	-	-	**88.79**	**14.95**
C2 (5.0mM for 12h)	8.33	75.00	16.67	9.09	72.73	18.18	-	100.00	-	-	88.89	11.11	**8.71**	**84.16**	**15.32**
C3 (7.5mM for 12h)	27.27	18.18	54.55	14.29	57.14	28.57	12.50	50.00	37.50	18.18	45.45	36.37	**18.06**	**42.69**	**39.25**
C4 (2.5mM for 24h)	11.11	11.11	77.78	25.00	12.50	62.50	11.11	-	88.89	14.29	-	85.71	**15.38**	**11.81**	**78.72**
C5 (2.5mM for 36h)	12.50	-	87.50	14.29	14.29	71.42	9.09	-	90.91	10.00	10.00	80.00	**11.47**	**12.15**	**82.46**
Mean	**14.80**	**47.50**	**50.16**	**15.67**	**49.67**	**37.80**	**10.90**	**75.93**	**59.88**	**14.16**	**61.09**	**53.30**	**13.88**	**58.54**	**50.28**

	4x *	2x **	Others ***
Stomatal density (No. of stomata mm^{-2})	**12.00 - 19.00**	**39.00 - 50.00**	**< 8.00 and mixoploids**
Stomatal dimension (length X breadth μ^{2})	**1850 - 2250**	**950 - 1200**	**> 2500 and mixoploids**

12h) were 8.33 and 9.09 for the cultivars Sannachenkadali and Anaikomban respectively. The percentages of plants with stomatal densities and dimensions characteristic of 'other' ploidy levels were 16.67, 18.18 and 11.11 for the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively.

Among the plants regenerated from cultures treated with C3 (7.5mM for 12h), 27.27%, 14.29%, 12.50% and 18.18% respectively of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan possessed tetraploid stomatal characters and 54.55%, 28.57%, 37.50% and 36.37% respectively possessed stomatal characters of other ploidy levels.

Among plants regenerated from cultures treated with C4 (2.5mM for 24h), 11.11%, 25.00%, 11.11% and 14.29% for the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had tetraploid stomatal traits and 77.78%, 62.50%, 88.89% and 85.71% respectively had stomatal traits characteristic of 'other' ploidy levels.

The percentages of plants with tetraploid stomatal characters among those regenarated from the cultures treated with C5 (2.5mM for 36h) were 12.50, 14.29, 9.09 and 10.00 respectively for the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan and those with 'other' ploidy stomatal characters were 87.50, 71.42, 90.91 and 80.00 respectively.

Among the regenerants, 14.80%, 15.67%, 10.90% and 14.16% derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had stomatal traits characteristic of tetraploids and 50.16%, 37.80%, 59.88% and 53.30% respectively had stomatal traits characteristic of 'other' ploids.

- **Plants regenerated from oryzalin-treated cultures**

The data (Table 46) revealed that among the plants regenerated from cultures treated with O1 (10μM for 3d), none fell under the tetraploid and 'other' categories. All the plants retained stomatal densities and dimensions characteristic of the original diploids.

Among the plants regenerated from cultures treated with O2 (20μM for 3d), 4.77%, 10.00% and 10.53% for the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively fell under the category of tetraploids. None of the plants of the cultivar. Kunnan had tetraploid stomatal characters. Plants possessing stomatal characters associated with the 'others' category were observed only for the cv. Thattillakunnan (5.26%).

Among the plants regenerated from cultures treated with O3 (30μM for 3d), 33.33%, 37.50%, 28.57% and 28.57% of plants of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively possessed stomatal densities and dimensions characteristic of tetraploids and 6.67%, 12.50%, 14.29% and 14.29% respectively had 'other' category of stomata.

The treatment O4 (40μM for 3d) resulted in 45.45%, 38.46 cent, 30.77% and 33.33% of plants of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively posessing tetraploid stomatal characters and 27.28%, 23.08%, 38.46% and 41.67% respectively possessing 'other' category stomata.

Among the plants regenerated from the cultures treated with O5 (10μM for 6d), 20.00%, 18.18%, 10.00% and 10.00% of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respetively had stomatal densities and dimensions characteristic of tetraploids and 20.00 each for the cultivars Sannachenkadali, Kunnan and Thattillakunnan respectively had

Table 46. Ploidy status (% of different ploidy levels) of regenerants derived from oryzalin-treated cultures as assessed by stomata analyses

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			Mean		
	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***
O1 (10μM for 3d)	-	100.00	-	-	100.00	-	-	100.00	-	-	100.00	-	**-**	**100.00**	**-**
O2 (20μM for 3d)	4.77	95.23	-	10.00	90.00	-	-	100.00	-	10.53	84.21	5.26	**8.43**	**92.36**	**5.26**
O3 (30μM for 3d)	33.33	60.00	6.67	37.50	50.00	12.50	28.57	57.14	14.29	28.57	57.14	14.29	**31.99**	**56.07**	**11.94**
O4 (40μM for 3d)	45.45	27.27	27.28	38.46	38.46	23.08	30.77	30.77	38.46	33.33	25.00	41.67	**37.00**	**30.38**	**32.62**
O5 (10μM for 6d)	20.00	60.00	20.00	18.18	81.82	-	10.00	70.00	20.00	10.00	60.00	30.00	**14.55**	**67.96**	**17.50**
O6 (20μM for 6d)	11.11	55.56	22.22	11.11	66.67	22.22	28.57	57.13	14.29	12.50	62.50	25.00	**15.82**	**60.47**	**20.93**
Mean	**22.93**	**66.34**	**19.04**	**23.05**	**71.16**	**14.45**	**24.48**	**69.17**	**21.76**	**18.99**	**64.81**	**23.24**	**22.36**	**67.87**	**19.62**

	4x *	**2x ****	**Others *****
Stomatal density (No. of stomata mm^{-2})	**12.00 - 19.00**	**39.00 - 50.00**	**< 8.00 and mixoploids**
Stomatal dimension (length X breadth μ^{2})	**1850 - 2250**	**950 - 1200**	**> 2500 and mixoploids**

,other, ploidy stomata. The cv. Anaikomban did not produce any plants belonging to the 'other' category.

The treatment O6 (20μM for 6d) resulted in 22.22%, 11.11%, 14.29% and 12.50% plants with tetraploid stomatal characters for the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively and 11.11%, 22.22%, 28.58% and 25.00% plants respectively with stomata characteristic of 'other' ploidy levels.

Among the regenerants, 22.93%, 23.05%, 24.48% and 18.99% derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had stomatal traits characteristic of tetraploids and 19.0.4%, 14.45%, 21.76% and 23.24% respectively had stomatal traits characteristic of 'other' ploids.

4.2.1.2. Chloroplast analyses (Tables 44, 47, 48; Plates 23 to 26)

Based on microscopic measurement of the density of chloroplasts (in terms of number of chloroplasts per guard cell pair of the stomata) in leaves of *in vitro* derived plants, the plants were ploidy-screened.

Plants with chloroplast densities of the ranges 9.00 – 11.00 and 15.00 – 17.00 chloroplasts guard cell pair^{-1} were categorised as diploids (Plate 25) and tetraploids (Plate 23 and 24) respectively. Those with chloroplast densities of 19.00 and above chloroplasts guard cell pair^{-1} were categorised as octoploids (Plates 25 and 26). Plants which had mixtures of two or more of these chloroplast densities were categorised as mixoploids. The octoploids and mixoploids were collectively categorised as 'others'.

The chloroplast traits of the *in vitro* derived plantlets are presented in Table 44.

➢ Plants regenerated from colchicine-treated cultures

The data (Table 47) revealed that none of the plants regenerated from the cultures treated with C1 (2.5mM for 12h) had chloroplast densities characteristic of tetraploids. The percentages of plants with chloroplast characters of 'other' ploids were 14.29, 8.33 and 22.22 for the cultivars Sannachenkadali, Anaikomban and Kunnan respectively.

The percentages of plants with tetraploid chloroplast characters among those regenerated from cultures treated with C2 (5.0mM for 12h) were 8.33 and 9.09 for the cultivars Sannachenkadali and Anaikomban respectively and those with chloroplast densities characteristic of 'other' ploidy levels were 16.67%, 18.18% and 11.11% for the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively.

Among the plants regenerated from cultures treated with C3 (7.5mM for 12h), 18.18%, 28.57%, 12.50% and 9.09% respectively of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan possessed tetraploid chloroplast characters and 45.45%, 14.29%, 37.50% and 36.36% respectively had chloroplast densities characteristic of other ploidy levels

Among plants regenerated from cultures treated with C4 (2.5mM for 24h), 22.22%, 12.50%, 11.11% and 14.29% for the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had tetraploid stomatal characters and 66.67%, 50.00%, 88.89% and 85.71% respectively had 'other' ploidy stomata characters.

Among the plants regenerated from cultures treated with C5 (2.5mM for 36h) 12.50%, 14.29%, 9.09% and 10.00% respectively for the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan had tetraploid chloroplast characters and 87.50%,

Table 47. Ploidy status (% of different ploidy levels) of regenerants derived from colchicine-treated cultures as assessed by chloroplast analyses

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			Mean		
	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***
C1 (2.5mM for 12h)	-	85.71	14.29	-	91.67	8.33	-	77.78	22.22	-	100.00	-	**-**	**88.79**	**14.95**
C2 (5.0mM for 12h)	8.33	75.00	16.67	9.09	72.73	18.18	-	100.00	-	-	88.89	11.11	**8.71**	**84.16**	**15.32**
C3 (7.5mM for 12h)	18.18	36.37	45.45	28.57	57.14	14.29	12.50	50.00	37.50	9.09	54.55	36.36	**17.09**	**49.52**	**33.40**
C4 (2.5mM for 24h)	22.22	11.11	66.67	12.50	37.50	50.00	11.11	-	88.89	14.29	-	85.71	**15.03**	**24.31**	**72.82**
C5 (2.5mM for 36h)	12.50	-	87.50	14.29	14.29	71.42	9.09	-	90.91	10.00	10.00	80.00	**11.47**	**12.15**	**82.46**
Mean	**15.31**	**52.05**	**46.12**	**16.11**	**54.67**	**32.44**	**10.90**	**75.93**	**59.88**	**11.13**	**63.36**	**53.30**	**13.36**	**61.50**	**47.93**

	4x *	2x **	Others ***
Chloroplast density (No. of chloroplasts guard cell pair^{-1})	**15.00 - 17.00**	**9.00 - 11.00**	**> 19.00 and mixoploids**

71.42%, 90.91% and 80.00% respectively possessed chloroplast densities characteristic of the 'other' ploidy levels.

Among the regenerants, 15.31%, 16.11%, 10.90% and 11.13% derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had stomatal traits characteristic of tetraploids and 46.12%, 32.44%, 59.88% and 53.30% respectively had stomatal traits characteristic of 'other' ploids.

- **Plants regenerated from oryzalin-treated cultures**

The data (Table 48) revealed that among the plants regenerated from cultures treated with O1 (10µM for 3d), none possessed chloroplast densities characteristic of tetraploid and 'other' categories. All the plants had chloroplast densities characteristic of diploids.

Among the plants regenerated from cultures treated with O2 (20µM for 3d), 4.77%, 10.00% and 10.52% for the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively fell under the category of tetraploids. None of the plants of the cv. Kunnan had tetraploid chloroplast characters. Plants possessing chloroplast characters associated with the 'others' category were observed only for the cv. Thattillakunnan (5.26%).

Among the plants regenerated from cultures treated with O3 (30µM for 3d), 26.67%, 25.00%, 28.57% and 21.43% of plants of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively possessed chloroplast densities characteristic of tetraploids and 6.66%, 6.25%, 14.29% and 14.29% respectively had 'other' category of chloroplasts.

The treatment O4 (40µM for 3d) resulted in 27.27%, 30.76 cent, 23.08% and 25.00% of plants of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively posessing tetraploid chloroplast characters and 9.09%,

Table 48. Ploidy status (% of different ploidy levels) of regenerants derived from oryzalin-treated cultures as assessed by chloroplast analyses

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			Mean		
	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***	4x *	2x * *	Others ***
O1 (10µM for 3d)	-	100.00	-	-	100.00	-	-	100.00	-	-	100.00	-	-	**100.00**	-
O2 (20µM for 3d)	4.77	95.23	-	10.00	90.00	-	-	100.00	-	10.52	84.22	5.26	**8.43**	**92.36**	**1.32**
O3 (30µM for 3d)	26.67	66.67	6.66	25.00	68.75	6.25	28.57	57.14	14.29	21.43	64.28	14.29	**25.42**	**64.21**	**10.37**
O4 (40µM for 3d)	27.27	63.64	9.09	30.76	38.47	30.77	23.08	46.15	30.77	25.00	25.00	50.00	**26.53**	**43.32**	**30.16**
O5 (10µM for 6d)	20.00	60.00	20.00	9.09	81.82	9.09	10.00	70.00	20.00	10.00	60.00	30.00	**12.27**	**67.96**	**19.77**
O6 (20µM for 6d)	22.22	55.56	22.22	11.11	66.67	22.22	14.29	71.43	14.28	12.50	62.50	25.00	**15.03**	**64.04**	**20.93**
Mean	**20.19**	**73.52**	**9.66**	**17.19**	**74.29**	**11.39**	**18.99**	**74.12**	**13.22**	**15.89**	**66.00**	**20.76**	**18.06**	**71.98**	**13.76**

	4x *	2x **	Others ***
Chloroplast density (No. of chloroplasts guard cell pair^{-1})	15.00 - 17.00	9.00 - 11.00	> 19.00 and mixoploids

30.77%, 30.77% and 50.00% respectively possessing the 'other' category chloroplast characters.

Among the plants regenerated from cultures treated with O5 (10µM for 6d), 20.00%, 9.09%, 10.00% and 10.00% of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respetively had chloroplast densities characteristic of tetraploids and 20.00%, 9.09%, 20.00% and 30.00% respectively had chloroplast characters belonging to the 'other' ploidy levels.

The treatment O6 (20µM for 6d) resulted in 22.22%, 11.11%, 14.29% and 12.50% plants with tetraploid chloroplast characters for the Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively and 22.22%, 22.22%, 14.28% and 25.00% plants with chloroplasts characteristic of the 'other' category ploidy levels.

Among the regenerants, 20.19%, 17.19%, 18.99% and 15.89% derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively had stomatal traits characteristic of tetraploids and 9.66%, 11.39%, 13.22% and 20.76% respectively had stomatal traits characteristic of 'other' ploids.

4.2.2. Confirmation of ploidy status (Tables 49 and 50; Plates 27 to 34)

The ploidy status of the *in vitro* regenerated plants as assessed based on stomatal and chloroplast traits were confirmed based on chromosome counts in the root tip cells.

Plants with 22 chromosomes ($2n = 2x = 22$) and 44 chromosomes ($2n = 4x = 44$) in their root tip cells were categorised as diploids (Plates 31 and 32) and tetraploids (Plates 27 to 30) respectively. A few octoploids ($2n = 2x = 88$) were also recorded (Plate 33), in which case the chromosomes could not be exactly counted as 88, but were corrected to the octoploid level, since they had chromosome numbers around 85-90. In many cases, root tip cells of the same plant were found to possess varying chromosome numbers such as (22 + 44), (22 + 88) and (44 + 88) (Plate 34). Such plants were also found to possess mixtures of varying stomatal densities, stomatal dimensions and chloroplast densities. These plants were categorised as mixoploids and the octoploids and mixoploids were collectively categorised as 'others'.

➢ Plants regenerated from colchicine-treated cultures

The data (Table 49) most of the plants regenerated from the cultures treated with C1 (2.5mM for 12h) maintained their original diploid status with a few of them expressing the 'other' ploidy status (14.29%, 8.33% and 22.22% for the cultivars Sannachenkadali, Anaikomban and Kunnan respectively), with the result that none of them turned out to be tetraploids.

The percentages of tetraploidy induced by the treatment C2 (5.0mM for 12h) were 8.33% and 9.09% in the cultivars Sannachenkadali and Anaikomban respectively. The percentages of 'others' were 16.67, 18.18 and 11.11% for the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively. The ploidy status of all the plants regenerated from the cv. Kunnan remained unaffected by the treatment C2 with the result that neither tetraploidy nor 'other' ploidy were recorded by the plants regenerated from Kunnan.

Among the plants regenerated from the cultures subjected to the treatment C3 (7.5mM for 12h), 18.18%, 28.57%, 12.50% and 9.09% of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively turned out to be tetraploids and 45.45%, 14.29%, 37.50% and 36.36% respectively fell under the 'other' category.

Table 49. Confirmation of ploidy status (% of different ploidy levels) of regenerants derived from colchicine-treated cultures based on chromosome counting

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			Mean		
	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	**4x** (2n=4x=44)	**2x** (2n=2x=22)	**Others** *
C1 (2.5mM for 12h)	-	85.71	14.29	-	91.67	8.33	-	77.78	22.22	-	100.00	-	-	**88.79**	**14.95**
C2 (5.0mM for 12h)	8.33	75.00	16.67	9.09	72.73	18.18	-	100.00	-	-	88.89	11.11	**8.71**	**84.16**	**15.32**
C3 (7.5mM for 12h)	18.18	36.37	45.45	28.57	57.14	14.29	12.50	50.00	37.50	9.09	54.55	36.36	**17.09**	**49.52**	**33.40**
C4 (2.5mM for 24h)	22.22	11.11	66.67	12.50	25.00	62.50	11.11	-	88.89	14.29	-	85.71	**15.03**	**18.06**	**75.94**
C5 (2.5mM for 36h)	12.50	-	87.50	14.29	14.29	71.42	9.09	-	90.91	10.00	10.00	80.00	**11.47**	**12.15**	**82.46**
Mean	**15.31**	**52.05**	**46.12**	**16.11**	**52.17**	**34.94**	**10.90**	**75.93**	**59.88**	**11.13**	**63.36**	**53.30**	**13.36**	**60.88**	**48.56**

Others * = Octoploids (2n=8x=88) + mixoploids

The rates of tetraploidy induced by the treatment C4 (2.5mM for 24h) in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan were 22.22%, 12.50%, 11.11% and 14 .29% respectively and the rates of 'other' ploidy levels were 66.67%, 62.50%, 88.89% and 85.71% respectively.

The treatment C5 (2.5mM for 36h) led to 12.50%, 14.29%, 9.09% and 10.00% tetraploidy and 87.50%, 71.42%, 90.91% and 80.00% 'other' ploidy in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively.

The average rates of tetraploidy induced by the treatments C1, C2, C3, C4 and C5 were 0.00%, 8.71%, 17.09%, 15.03% and 11.47% respectively, and the average percentages of plants falling under the category 'others' were 14.95%, 15.32%, 33.40%, 75.94% and 82.46% respectively. The cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan recorded 15.31%, 16.11%, 10.90% and 11.13% tetraploidy respectively.

Finally, the overall averages of the rates of tetraploidy and the 'other' category induced by the colchine treatments were 13.36% and 48.56% respectively.

➢ Plants regenerated from oryzalin-treated cultures

The data (Table 50) revealed that the ploidy status of all the plants regenerated from the cultures treated with O1 (10μM for 3d) remained unaffected.

The percentages of tetraploidy induced by the treatment O2 (20μM for 3d) were 4.77, 10.00 and 10.52 in the cultivars Sannachenkadali, Anaikomban and Thattillakunnan respectively. The treatment O2 (20μM for 3d) induced no plants belonging to the 'other' category except for a very low frequency of 5.26% induced in the cv. Thattillakunnan. The ploidy status of all the plants regenerated from the cv. Kunnan remained unaffected by

Table 50. Confirmation of ploidy status (% of different ploidy levels) of regenerants derived from oryzalin-treated cultures based on chromosome counting

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan			**Mean**		
	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	4x (2n=4x=44)	2x (2n=2x=22)	Others *	**4x** (2n=4x=44)	**2x** (2n=2x=22)	**Others ***
O1 (10µM for 3d)	-	100.00	-	-	100.00	-	-	100.00	-	-	100.00	-	-	**100.00**	-
O2 (20µM for 3d)	4.77	95.23	-	10.00	90.00	-	-	100.00	-	10.52	84.22	5.26	**8.43**	**92.36**	**1.32**
O3 (30µM for 3d)	26.67	66.67	6.66	25.00	68.75	6.25	21.43	57.14	21.43	21.43	64.28	14.29	**23.63**	**64.21**	**12.16**
O4 (40µM for 3d)	27.27	63.64	9.09	30.76	38.47	30.77	23.08	46.15	30.77	25.00	25.00	50.00	**26.53**	**43.32**	**30.16**
O5 (10µM for 6d)	20.00	60.00	20.00	9.09	90.91	-	10.00	70.00	20.00	10.00	60.00	30.00	**12.27**	**70.23**	**17.50**
O6 (20µM for 6d)	22.22	55.56	22.22	11.11	66.67	22.22	14.29	71.43	14.28	12.50	62.50	25.00	**15.03**	**64.04**	**20.93**
Mean	**20.19**	**73.52**	**9.66**	**17.19**	**75.80**	**9.87**	**17.20**	**74.12**	**14.41**	**15.89**	**66.00**	**20.76**	**17.62**	**72.36**	**13.68**

Others * = Octoploids (2n=8x=88) + mixoploids the treatment O2 (10μM for 3d) with the result that neither tetraploidy nor 'other' ploidy were recorded by the plants regenerated from Kunnan.

Among the plants regenerated from the cultures subjected to the treatment O3 (30μM for 3d), 26.67%, 25.00%, 21.43% and 21.43% of plants of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively turned out to be tetraploids and 6.66%, 6.25%, 21.43% and 14.29% respectively were of the 'other' category.

The rates of tetraploidy induced by the treatment O4 (40μM for 3d) in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan were 27.27%, 30.76%, 23.08% and 25.00% respectively and the rates of 'other' ploidy levels were 9.09%, 30.77%, 30.77% and 50.00% respectively.

The treatment O5 (10μM for 6d) led to 20.00%, 9.09%, 10.00% and 10.00% tetraploidy in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively and 20.00%, 20.00% and 30.00% 'other' ploidy in the cultivars Sannachenkadali, Kunnan and Thattillakunnan respectively.

The treatment O6 (20μM for 6d) induced 22.22%, 11.11%, 14.29% and 12.50% tetraploidy and 22.22%, 22.22%, 14.28% and 25.00% 'other' ploids among the plants regenerated from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively.

The average percentages of tetraploidy induced by the treatments O1, O2, O3, O4, O5 and O6 were 0.00, 8.43, 23.63, 26.53, 12.27 and 15.03 respectively. The average percentages of plants falling under the 'others' category were 0.00, 1.32, 12.16, 30.16, 17.50 and 20.93 respectively. The cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan recorded 20.19%, 17.19%, 17.20% and 15.89% tetraploidy respectively.

Finally, the overall averages of the rates of tetraploiy and the 'other' category were 17.62% and 13.68% respectively.

- **Colchicine Vs oryzalin**

The overall average percentage of tetraploidy induced by colchicine was 13.36% and it was lower than that of oryzalin (17.62%). The overall average percentages of plants belonging to the 'others' category were 48.56% for colchicine and 13.68% for oryzalin respectively.

4.2.3. Degree of reliability of the ploidy assessment methods

(Table 51; Fig.14)

Although most of the results of the ploidy-screening experiments based on stomata and chloroplast analyses agreed with those of the confirmatory analyses performed through chromosome counting, a few of them were proved contradictory by the chromosome counts (Table 51).

Based on these results, the degree of reliability was calculated for each of the two methods by comparing with the chromosome counting method which the most reliable ploidy assessment method. The degrees of reliability were 82.19% and 95.89% respectively for ploidy assessment through stomata analyses and chloroplast analyses (Fig. 14).

<u>Stomata analyses</u>

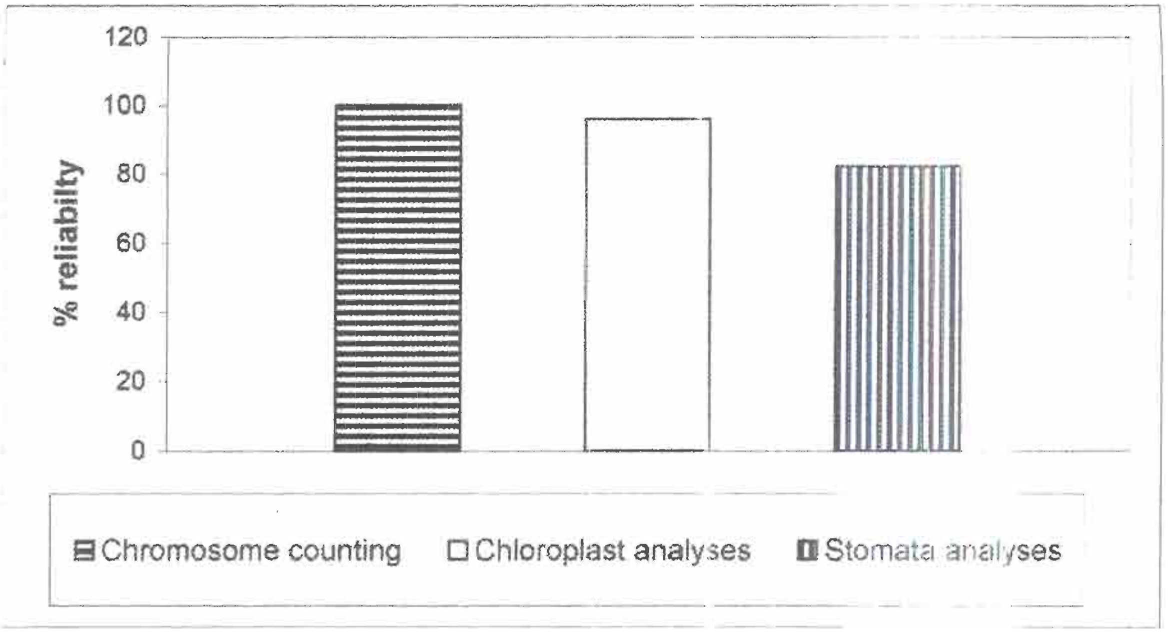
Total number of plants subjected to ploidy screening - 73
Number of contradicting cases - 13
Reliability of ploidy assessment through stomata analyses- 82.19%

Table 51. Contradictory results in ploidy assessment by various methods

Treatment	Regenerant No	Ploidy status as assessed by stomata analyses	Ploidy status as assessed by chloroplast analyses	Ploidy status as confirmed by chromosome counting
Colchicine				
cv Shannachenkadali				
C3	9	4x	2x	2x
C4	4	Mixoploidy	4x	4x
cv Annaikomban				
C3	3	Mixoploidy	4x	4x
C4	**7**	**Mixoploidy**	**2x**	**Mixoploidy**
cv Thattillakunnan				
C3	9	4x	Mixoploidy	Mixoploidy
Oryzalin				
cv Sannachenkadali				
O3	9	4x	2x	2x
O6	4	Mixoploidy	4x	4x
cv Anaikomban				
O4	13	Mixoploidy	2x	2x
O5	**4**	**2x**	**Mixoploidy**	**2x**
	7	4x	2x	2x
cv Kunnan				
O3	***10***	***4x***	***4x***	***2x***
O4	7	4x	2x	2x
O6	6	4x	2x	2x
cv Tattillakunnan				
O3	11	4x	2x	2x
O4	1	4x	2x	2x

Note : **Bold**	-	Chloroplast analysis contradicting chromosome count
Bold Italics	-	Both stomata and chloroplast analyses contradicting chromosome count
Others	-	Only stomata analyses contradicting chromosome count

Fig. 17. Degree of reliability of ploidy assessment methods



Chloroplast analyses

Total number of plants subjected to ploidy screening	- 73
Number of contradicting cases	- 3
Reliability of ploidy assessment by chloroplast analyses	- 95.89%

4.3. EVALUATION OF *IN VITRO* INDUCED TETRAPLOIDS

4.3.1. Effect of tetraploidy on morphological parameters

4.3.1.1. Pseudostem height (Tables 52a and b; Fig.9; Plates 35 to 38)

At the end of the hardening phase, when the induced tetraploids were ready for transfer to *ex vitro* environment, they had shorter pseudostems than the corresponding diploids (Tables 52a and b; Plates 35 to 38). The tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan recorded pseudostem heights ranging between 7.21cm (S-Tetra 3) and 10.14cm (S-Tetra 7), 7.18cm (A-Tetra 16) and 10.21cm (A-Tetra 10), 7.39cm (K-Tetra 7) and 10.24cm (K-Tetra 1) and 7.33cm (T-Tetra 12) and 10.14cm (T-Tetra 1). The values for the corresponding diploids were 15.13cm ,16.24cm, 17.18cm and 17.34cm.

Two months after transfer to *ex vitro* environment, the pseudostems of the induced tetraploids remained shorter than those of the diploids. The values for the tetraploids derived from the cv. Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 23.14cm (S-Tetra 15) and 26.49cm (S-Tetra 8), 23.36cm (A-Tetra 5) and 27.93cm (A-Tetra 14), 24.28cm (K-Tetra 6) and 27.83cm (S-Tetra 10) and 24.19cm (T-Tetra 7) and 27.73cm (T-Tetra 9) respectively. The values for the respective diploids were 38.15cm, 38.08cm, 39.23cm and 39.46cm.

Four months after transfer to *ex vitro* environment, the pseudostems of the induced tetraploids had attained greater

Table 52(a). **Pseudostem height (cm) of the *in vitro* induced tetraploids derived from the cultivars Sannachenkadali and Anaikomban**

Treatment		Sannachenkadali						Anaikomban						
	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C2	S-Tetra 1	8.23	-45.60	24.50	-35.78	74.22	9.92	A-Tetra 1	8.36	-48.52	25.93	-31.91	74.16	8.83
C3	S-Tetra 2	8.94	-40.91	24.31	-36.28	74.13	9.79	A-Tetra 2	8.14	-49.88	24.36	-36.03	74.23	8.94
	S-Tetra 3	7.21	-52.35	24.19	-36.59	73.29	8.55	A-Tetra 3	7.31	-54.99	25.22	-33.77	74.48	9.30
C4	S-Tetra 4	8.48	-43.95	25.20	-33.94	73.33	8.60	A-Tetra 4	7.24	-55.42	24.19	-36.48	73.39	7.70
	S-Tetra 5	8.78	-41.97	24.14	-36.72	72.64	7.58							
C5	S-Tetra 6	8.26	-45.41	24.23	-36.49	73.18	8.38	A-Tetra 5	8.16	-49.75	23.36	-38.66	72.68	6.66
Oryzalin														
O2	S-Tetra 7	10.14	-32.98	26.28	-31.11	76.14	12.77	A-Tetra 6	7.33	-54.86	24.28	-36.24	76.39	12.11
								A-Tetra 7	7.24	-55.42	24.73	-35.06	76.71	12.58
O3	S-Tetra 8	10.07	-33.44	26.49	-30.56	75.23	11.42	A-Tetra 8	9.08	-44.09	25.16	-33.93	75.24	10.42
	S-Tetra 9	9.36	-38.14	24.31	-36.28	75.31	11.54	A-Tetra 9	10.13	-37.62	26.77	-29.70	76.14	11.74
	S-Tetra 10	9.18	-39.33	23.28	-38.98	74.22	9.92	A-Tetra 10	10.21	-37.13	26.82	-29.57	75.31	10.52
	S-Tetra 11	9.36	-38.14	23.62	-38.09	74.16	9.83	A-Tetra 11	10.16	-37.44	26.34	-30.83	74.26	8.98
O4	S-Tetra 12	9.89	-34.63	24.16	-36.67	75.08	11.20	A-Tetra 12	9.42	-42.00	27.19	-28.60	74.93	9.96
	S-Tetra 13	8.43	-44.28	24.28	-36.36	75.31	11.54	A-Tetra 13	8.33	-48.71	26.22	-31.14	73.86	8.39
	S-Tetra 14	8.68	-42.63	24.03	-37.01	74.26	9.98	A-Tetra 14	8.19	-49.57	27.93	-26.65	74.28	9.01
O5	S-Tetra 15	8.12	-46.33	23.14	-39.34	74.31	10.06	A-Tetra 15	7.26	-55.30	27.64	-27.42	74.43	9.23
O6	S-Tetra 16	8.06	-46.73	23.22	-39.13	75.73	12.16	A-Tetra 16	7.18	-55.79	26.36	-30.78	74.21	8.91
Range		**7.21 - 10.14**		**23.14 - 26.49**		**72.64 - 76.14**			**7.18 - 10.21**		**23.36 - 27.93**		**72.68 - 76.71**	
Control		15.13		38.15		67.52			16.24		38.08		68.14	

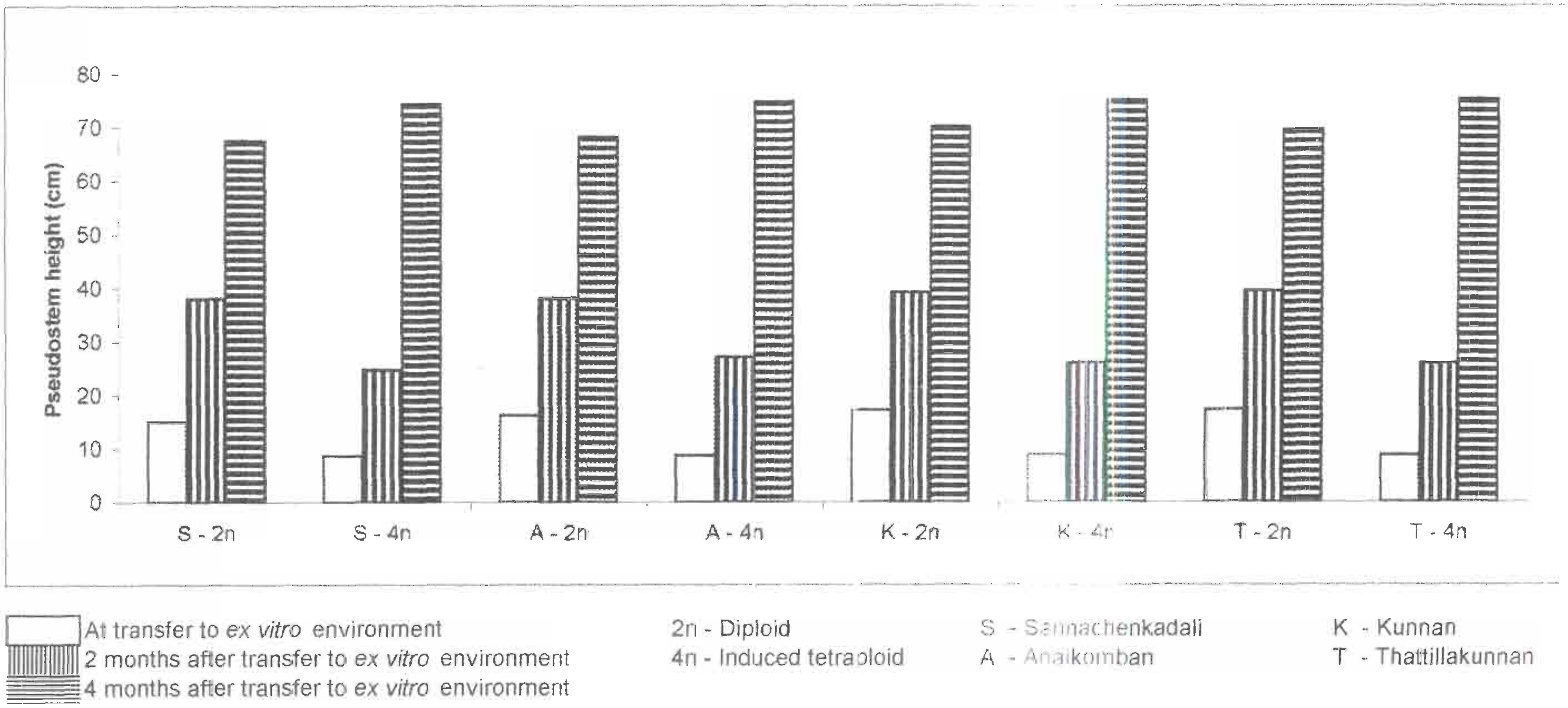
* MAT - Months after transfer to *ex vitro* environment

Table 52(b). Pseudostem height (cm) of the *in vitro* induced tetraploids derived from the cultivars Kunnan and Thattillakunnan

Treatment	Kunnan							Thattillakunnan						
	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C3	K-Tetra 1	10.24	-40.40	25.33	-35.43	73.14	4.40	T-Tetra 1	10.14	-41.52	25.16	-36.24	75.16	8.21
C4	K-Tetra 2	10.16	-40.86	26.12	-33.42	73.22	4.51	T-Tetra 2	8.63	-50.23	24.33	-38.34	74.23	6.87
C5	K-Tetra 3	9.38	-45.40	25.73	-34.41	74.97	7.01	T-Tetra 3	9.29	-46.42	26.19	-33.63	74.06	6.62
Oryzalin														
O2	K-Tetra 4	8.19	-52.33	24.33	-37.98	76.82	9.65	T-Tetra 4	8.14	-53.06	27.63	-29.98	76.29	9.83
								T-Tetra 5	9.23	-46.77	25.82	-34.57	76.43	10.03
O3	K-Tetra 5	8.22	-52.15	26.19	-33.24	76.19	8.75	T-Tetra 6	9.06	-47.75	24.36	-38.27	77.14	11.06
	K-Tetra 6	9.16	-46.68	24.28	-38.11	76.28	8.88	T-Tetra 7	8.79	-49.31	24.19	-38.70	74.78	7.66
	K-Tetra 7	7.39	-56.98	24.38	-37.85	75.32	7.51	T-Tetra 8	8.38	-51.67	25.28	-35.94	74.39	7.10
O4	K-Tetra 8	8.45	-50.81	25.13	-35.94	76.18	8.74	T-Tetra 9	7.42	-57.21	27.73	-29.73	74.82	7.72
	K-Tetra 9	8.79	-48.84	25.46	-35.10	73.44	4.82	T-Tetra 10	8.19	-52.77	26.16	-33.71	73.16	5.33
	K-Tetra 10	9.31	-45.81	27.83	-29.06	74.26	5.99	T-Tetra 11	8.26	-52.36	27.33	-30.74	73.46	5.76
O5	K-Tetra 11	9.06	-47.26	26.19	-33.24	74.73	6.67	T-Tetra 12	7.33	-57.73	27.26	-30.92	74.28	6.94
	K-Tetra 12	8.22	-52.15	26.39	-32.73	74.18	5.88							
O6								T-Tetra 13	8.76	-49.48	27.14	-31.22	74.11	6.69
Range		**7.39 - 10.24**		**24.28 - 27.83**		**73.14 - 76.82**			**7.33 - 10.14**		**24.19 - 27.73**		**73.16 - 77.14**	
Control		17.18		39.23		70.06			17.34		39.46		69.46	

* MAT - Months after transfer to *ex vitro* environment

Fig 9. Pseudostem height of induced tetraploids and their corresponding diploids



heights than those of the corresponding diploids, thus indicating a very rapid growth following the initial retarded growth. The values for the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 72.64cm (S-Tetra 5) and 76.14cm (S-Tetra 7), 72.68cm (A-Tetra 5) and 76.71cm (A-Tetra 7), 73.14cm (K-Tetra 1) and 76.82cm (S-Tetra 4) and 73.16cm (T-Tetra 10) and 77.14cm (T-Tetra 6) respectively. The respective diploids recorded pseudostem heights of 67.52cm, 68.14cm, 70.06cm 69.46cm respectively.

4.3.1.2. Pseudostem girth (Tables 53a and b; Plates 35 to 38)

At transfer to *ex vitro* environment, the induced tetraploids had thinner stems than their respective diploids (Tables 53a and b; Plates 35 to 38). The pseudostem girth of the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 2.63cm (S-Tetra 14) and 3.22cm (S-Tetra 7), 2.73cm (A-Tetra 13) and 3.12cm (A-Tetra 6), 2.14cm (K-Tetra 2) and 3.36cm (S-Tetra 4) and 2.73cm (T-Tetra 13) and 3.31cm (T-Tetra 4) respectively. The values for their respective diploids were 4.20cm, 4.40cm, 4.75cm and 4.63cm.

Two months after transfer to *ex vitro* environment, the pseudostems of the induced tetraploids remained thinner than those of the corresponding diploids. The values for the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 7.06cm (S-Tetra 4) and 8.79cm (S-Tetra 10), 7.22cm (A-Tetra 9) and 8.49cm (A-Tetra 6), 7.33cm (K-Tetra 3) and 9.26cm (S-Tetra 5) and 7.29cm (T-Tetra 12) and 9.66cm (T-Tetra 4) respectively. The values for their respective diploids were 9.26cm, 9.24cm, 9.89cm and 10.12cm.

Four months after transfer to *ex vitro* environment, the pseudostems of the induced tetraploids had grown thicker than

Table 53(a). Pseudostem girth (cm) of the *in vitro* induced tetraploids derived from the cultivars Sannachenkadali and Anaikomban

	Sannachenkadali							Anaikomban						
Treatment	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C2	S-Tetra 1	3.12	-25.71	8.45	-8.75	20.42	25.74	A-Tetra 1	3.08	-30.00	7.23	-21.75	21.32	31.36
C3	S-Tetra 2	3.09	-26.43	8.23	-11.12	21.31	31.22	A-Tetra 2	3.02	-31.36	7.53	-18.51	20.14	24.09
	S-Tetra 3	3.02	-28.10	7.28	-21.38	20.64	27.09	A-Tetra 3	2.93	-33.41	8.17	-11.58	20.23	24.65
C4	S-Tetra 4	2.86	-31.90	7.06	-23.76	22.13	36.27	A-Tetra 4	2.80	-36.36	7.46	-19.26	22.16	36.54
	S-Tetra 5	2.79	-33.57	7.29	-21.27	22.41	37.99							
C5	S-Tetra 6	2.71	-35.48	7.21	-22.14	21.08	29.80	A-Tetra 5	2.87	-34.77	7.34	-20.56	19.18	18.18
Oryzalin														
O2	S-Tetra 7	3.22	-23.33	8.44	-8.86	22.31	37.38	A-Tetra 6	3.12	-29.09	8.49	-8.12	21.14	30.25
								A-Tetra 7	3.09	-29.77	8.47	-8.33	23.26	43.31
O3	S-Tetra 8	3.10	-26.19	8.36	-9.72	22.14	36.33	A-Tetra 8	2.96	-32.73	8.36	-9.52	20.19	24.40
	S-Tetra 9	3.15	-25.00	8.14	-12.10	21.61	33.07	A-Tetra 9	2.84	-35.45	7.22	-21.86	22.04	35.80
	S-Tetra 10	2.90	-30.95	8.79	-5.08	20.14	24.01	A-Tetra 10	2.93	-33.41	7.65	-17.21	20.13	24.03
	S-Tetra 11	3.09	-26.43	7.21	-22.14	22.21	36.76	A-Tetra 11	2.86	-35.00	7.25	-21.54	20.24	24.71
O4	S-Tetra 12	2.93	-30.24	7.29	-21.27	23.19	42.80	A-Tetra 12	2.81	-36.14	7.47	-19.16	21.16	30.38
	S-Tetra 13	2.84	-32.38	8.26	-10.80	23.16	42.61	A-Tetra 13	2.73	-37.95	7.22	-21.86	20.33	25.26
	S-Tetra 14	2.63	-37.38	7.59	-18.03	21.24	30.79	A-Tetra 14	3.01	-31.59	7.39	-20.02	19.28	18.79
O5	S-Tetra 15	2.79	-33.57	7.08	-23.54	22.08	35.96	A-Tetra 15	2.93	-33.41	7.48	-19.05	22.43	38.20
O6	S-Tetra 16	2.83	-32.62	7.43	-19.76	21.12	30.05	A-Tetra 16	2.84	-35.45	7.39	-20.02	21.62	33.21
Range		**2.63 - 3.22**		**7.06 - 8.79**		**20.14 - 23.19**			**2.73 - 3.12**		**7.22 - 8.49**		**19.18 - 23.26**	
Control		4.20		9.26		16.24			4.40		9.24		16.23	

* MAT - Months after transfer to *ex vitro* environment

Table 53(b). Pseudostem girth (cm) of the *in vitro* induced tetraploids derived from the cultivars Kunnan and Thattillakunnan

Treatment	Kunnan							Thattillakunnan						
	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C3	K-Tetra 1	3.06	-35.58	8.36	-15.47	23.16	19.50	T-Tetra 1	3.14	-32.18	9.63	-4.84	22.09	19.86
C4	K-Tetra 2	2.14	-54.95	8.41	-14.96	22.14	14.24	T-Tetra 2	2.90	-37.37	8.24	-18.58	24.69	33.97
C5	K-Tetra 3	2.81	-40.84	7.33	-25.88	24.03	23.99	T-Tetra 3	2.88	-37.80	8.38	-17.19	24.32	31.96
Oryzalin														
O2	K-Tetra 4	3.36	-29.26	9.19	-7.08	25.22	30.13	T-Tetra 4	3.31	-28.51	9.66	-4.55	23.18	25.77
								T-Tetra 5	3.26	-29.59	9.41	-7.02	25.03	35.81
O3	K-Tetra 5	3.12	-34.32	9.26	-6.37	24.39	25.85	T-Tetra 6	3.14	-32.18	8.29	-18.08	22.73	23.33
	K-Tetra 6	3.19	-32.84	9.14	-7.58	24.14	24.56	T-Tetra 7	2.93	-36.72	9.38	-7.31	25.42	37.93
	K-Tetra 7	2.98	-37.26	8.56	-13.45	23.61	21.83	T-Tetra 8	2.98	-35.64	8.37	-17.29	26.18	42.05
O4	K-Tetra 8	2.84	-40.21	8.23	-16.78	25.72	32.71	T-Tetra 9	2.86	-38.23	8.69	-14.13	24.32	31.96
	K-Tetra 9	3.04	-36.00	7.93	-19.82	25.18	29.93	T-Tetra 10	2.81	-39.31	8.08	-20.16	24.16	31.09
	K-Tetra 10	3.15	-33.68	8.12	-17.90	24.39	25.85	T-Tetra 11	2.79	-39.74	7.46	-26.28	23.38	26.86
O5	K-Tetra 11	2.86	-39.79	7.34	-25.78	26.83	38.44	T-Tetra 12	2.81	-39.31	7.29	-27.96	22.14	20.13
	K-Tetra 12	2.93	-38.32	7.63	-22.85	24.11	24.41							
O6								T-Tetra 13	2.73	-41.04	7.31		24.08	
Range		**2.14 - 3.36**		**7.33 - 9.26**		**22.14 - 26.83**			**2.73 - 3.31**		**7.29 - 9.66**		**22.09 - 26.18**	
Control		4.75		9.89		19.38			4.63		10.12		18.43	

* MAT - Months after transfer to *ex vitro* environment

those of the corresponding diploids, thus indicating a very rapid growth following the initial retarded growth. The values for the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 20.14cm (S-Tetra 10) and 23.19cm (S-Tetra 12), 19.18cm (A-Tetra 5) and 23.26cm (A-Tetra 7), 22.14cm (K-Tetra 2), 26.83cm (S-Tetra 11) and 22.09cm (T-Tetra 1) and 26.18cm (T-Tetra 8). The values for the respective diploids were 16.24cm, 16.23cm, 19.38cm and 18.43cm.

4.3.1.3. **Leaf area** (Table 54)

All the induced tetraploids had larger leaves than the corresponding diploids (Table 54, Plates 35 to 38). The ranges of the values for leaf area of the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan were 0.029m^2 - 0.036m^2, 0.029m^2 - 0.033m^2, 0.031m^2 - 0.041m^2 and 0.032m^2 - 0.038m^2 respectively. The values for the respective diploids were 0.021m^2 , 0 .021m^2, 0.022m^2 and 0.023m^2.

4.3.2. **Effect of tetraploidy on physiological parameters**

4.3.2.1. **Phyllochron** (Tables 55a and b; Fig. 10)

All the induced tetraploids, irrespective of the cv. from which they were derived and the stage of growth, took longer duration for leaf emergence, with the result that the phyllochron values for all the tetraploids were grater than those of the corresponding diploids (Tables 55a and b). With progress in growth, the phyllochron values of the tetraploids greatly reduced, indicating faster growth rates during the later stages.

At transfer to *ex vitro* environment, the phyllochron values of the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 14.00

Table 54. Leaf area of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	Leaf area (m^2)	% increase over control	Name of tetraploid	Leaf area (m^2)	% increase over control	Name of tetraploid	Leaf area (m^2)	% increase over control	Name of tetraploid	Leaf area (m^2)	% increase over control
Colchicine												
C2	S-Tetra1	0.031	46.67	A-tetra1	0.031	49.52						
C3	S-Tetra2	0.030	40.95	A-Tetra2	0.033	57.62	K-Tetra1	0.032	45.91	T-Tetra1	0.032	40.87
	S-Tetra3	0.029	40.00	A-Tetra3	0.033	55.24						
C4	S-Tetra4	0.032	53.81	A-Tetra4	0.032	51.43	K-Tetra2	0.033	51.82	T-Tetra2	0.036	57.39
	S-Tetra5	0.031	48.57									
C5	S-Tetra6	0.030	44.29	A-Tetra5	0.031	47.14	K-Tetra3	0.039	75.45	T-Tetra3	0.032	38.70
Oryzalin												
O2	S-Tetra7	0.032	54.29	A-Tetra6	0.031	48.57	K-Tetra4	0.034	55.00	T-Tetra4	0.038	65.65
				A-Tetra7	0.029	37.62				T-Tetra5	0.037	62.17
O3	S-Tetra8	0.033	55.24	A-Tetra8	0.033	55.24	K-Tetra5	0.037	67.73	T-Tetra6	0.034	48.70
	S-Tetra9	0.030	40.95	A-Tetra9	0.031	49.52	K-Tetra6	0.041	85.45	T-Tetra7	0.032	37.39
	S-Tetra10	0.032	51.90	A-Tetra10	0.033	58.10	K-Tetra7	0.033	48.18	T-Tetra8	0.037	59.13
	S-Tetra11	0.032	53.81	A-Tetra11	0.033	55.24						
O4	S-Tetra12	0.033	55.24	A-Tetra12	0.030	41.90	K-Tetra8	0.034	55.45	T-Tetra9	0.034	48.70
	S-Tetra13	0.033	59.05	A-Tetra13	0.032	54.29	K-Tetra9	0.032	43.64	T-Tetra10	0.034	46.96
	S-Tetra14	0.033	57.62	A-Tetra14	0.029	36.19	K-Tetra10	0.032	45.91	T-Tetra11	0.032	38.70
O5	S-Tetra15	0.031	48.57	A-Tetra15	0.031	48.57	K-Tetra11	0.035	58.18	T-Tetra12	0.032	40.87
							K-Tetra12	0.031	42.73			
O6	S-Tetra16	0.036	73.33	A-Tetra16	0.032	52.86				T-Tetra13	0.032	37.39
Range		**0.029 - 0.036**			**0.029 - 0.033**			**0.031 - 0.041**			**0.032 - 0.038**	
Control		0.021			0.021			0.022			0.023	

Table 55(a). Phyllochron (days) of the *in vitro* induced tetraploids derived from the cultivars Sannachenkadali and Anaikomban

Treatment		Sannachenkadali						Anaikomban						
	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C2	S-Tetra 1	16.00	72.41	14.00	89.70	8.00	18.69	A-Tetra 1	16.00	65.46	14.00	79.26	8.00	16.96
C3	S-Tetra 2	16.00	72.41	14.00	89.70	8.00	18.69	A-Tetra 2	16.00	65.46	14.00	79.26	8.00	16.96
	S-Tetra 3	16.00	72.41	14.00	89.70	9.00	33.53	A-Tetra 3	17.00	75.80	13.00	66.45	7.00	2.34
C4	S-Tetra 4	16.00	72.41	14.00	89.70	9.00	33.53	A-Tetra 4	17.00	75.80	14.00	79.26	8.00	16.96
	S-Tetra 5	17.00	83.19	14.00	89.70	9.00	33.53							
C5	S-Tetra 6	17.00	83.19	14.00	89.70	10.00	48.37	A-Tetra 5	17.00	75.80	15.00	92.06	8.00	16.96
Oryzalin														
O2	S-Tetra 7	15.00	61.64	13.00	76.15	8.00	18.69	A-Tetra 6	15.00	55.12	13.00	66.45	7.00	2.34
								A-Tetra 7	15.00	55.12	13.00	66.45	7.00	2.34
O3	S-Tetra 8	15.00	61.64	13.00	76.15	8.00	18.69	A-Tetra 8	15.00	55.12	13.00	66.45	7.00	2.34
	S-Tetra 9	15.00	61.64	13.00	76.15	8.00	18.69	A-Tetra 9	16.00	65.46	14.00	79.26	7.00	2.34
	S-Tetra 10	15.00	61.64	14.00	89.70	8.00	18.69	A-Tetra 10	16.00	65.46	14.00	79.26	8.00	16.96
	S-Tetra 11	15.00	61.64	14.00	89.70	8.00	18.69	A-Tetra 11	16.00	65.46	14.00	79.26	8.00	16.96
O4	S-Tetra 12	14.00	50.86	14.00	89.70	8.00	18.69	A-Tetra 12	16.00	65.46	14.00	79.26	8.00	16.96
	S-Tetra 13	15.00	61.64	14.00	89.70	9.00	33.53	A-Tetra 13	16.00	65.46	13.00	66.45	8.00	16.96
	S-Tetra 14	16.00	72.41	14.00	89.70	9.00	33.53	A-Tetra 14	15.00	55.12	13.00	66.45	7.00	2.34
O5	S-Tetra 15	16.00	72.41	15.00	103.25	9.00	33.53	A-Tetra 15	16.00	65.46	14.00	79.26	7.00	2.34
O6	S-Tetra 16	16.00	72.41	15.00	103.25	9.00	33.53	A-Tetra 16	16.00	65.46	14.00	79.26	8.00	16.96
Range		**14 - 17**		**13 - 15**		**8 - 10**			**15 - 17**		**13 - 15**		**7 - 8**	
Control		9.28		7.38		6.74			9.67		7.81		6.84	

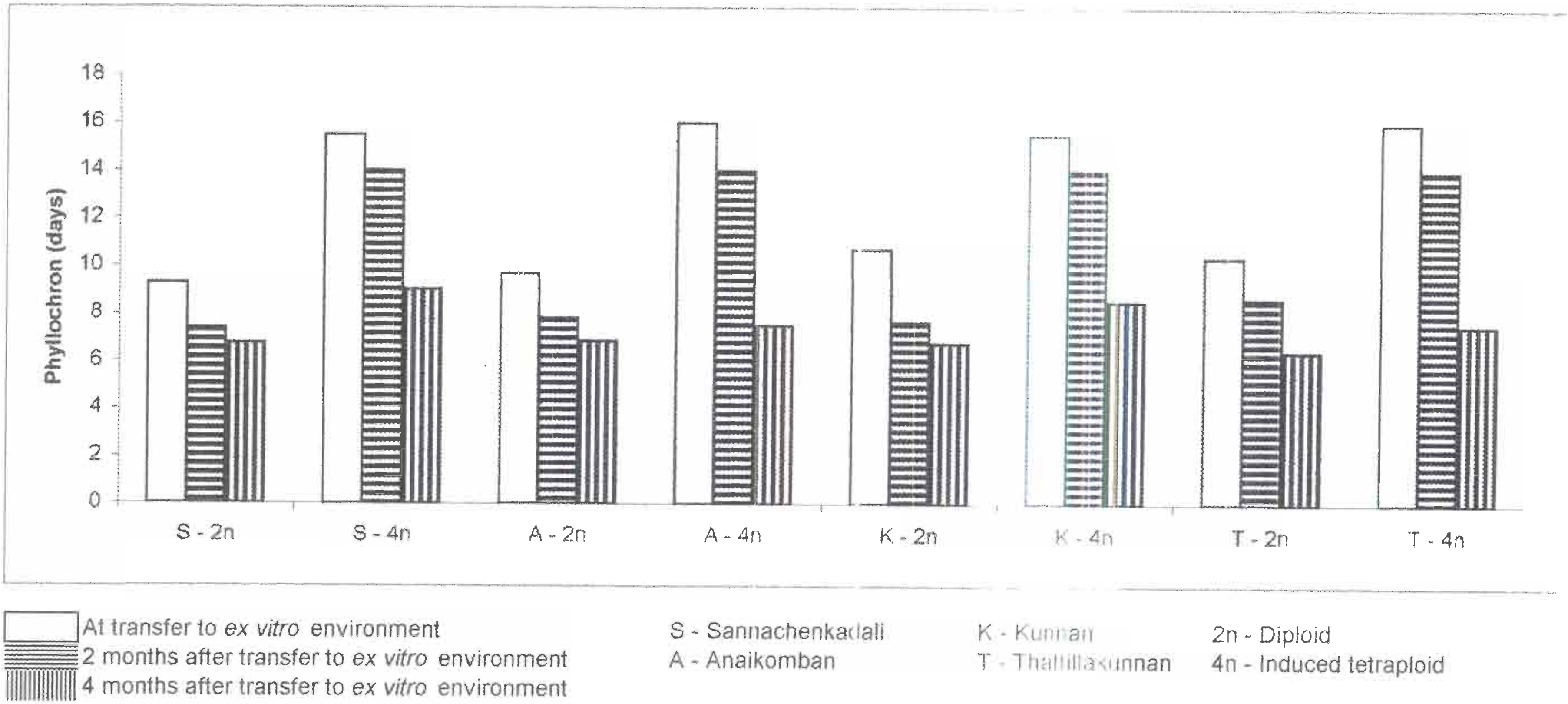
* MAT - Months after transfer to *ex vitro* environment

Table 55(b). **Phyllochron (days) of the *in vitro* induced tetraploids derived from the cultivars Kunnan and Thattillakunnan**

Treatment	Kunnan							Thattillakunnan						
	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	End of *in vitro* phase	% difference over control	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine														
C3	K-Tetra 1	16.00	49.81	14.00	83.49	9.00	33.53	T-Tetra 1	16.00	54.44	14.00	62.22	8.00	24.42
C4	K-Tetra 2	16.00	49.81	15.00	96.59	9.00	33.53	T-Tetra 2	17.00	64.09	14.00	62.22	8.00	24.42
C5	K-Tetra 3	17.00	59.18	15.00	96.59	8.00	18.69	T-Tetra 3	17.00	64.09	15.00	73.81	7.00	8.86
Oryzalin														
O2	K-Tetra 4	14.00	31.09	13.00	70.38	8.00	18.69	T-Tetra 4	15.00	44.79	13.00	50.64	8.00	24.42
								T-Tetra 5	15.00	44.79	13.00	50.64	8.00	24.42
O3	K-Tetra 5	14.00	31.09	14.00	83.49	8.00	18.69	T-Tetra 6	15.00	44.79	14.00	62.22	7.00	8.86
	K-Tetra 6	15.00	40.45	14.00	83.49	8.00	18.69	T-Tetra 7	15.00	44.79	14.00	62.22	7.00	8.86
	K-Tetra 7	16.00	49.81	13.00	70.38	9.00	33.53	T-Tetra 8	16.00	54.44	14.00	62.22	7.00	8.86
O4	K-Tetra 8	16.00	49.81	15.00	96.59	9.00	33.53	T-Tetra 9	16.00	54.44	14.00	62.22	8.00	24.42
	K-Tetra 9	16.00	49.81	14.00	83.49	8.00	18.69	T-Tetra 10	16.00	54.44	14.00	62.22	8.00	24.42
	K-Tetra 10	16.00	49.81	14.00	83.49	9.00	33.53	T-Tetra 11	16.00	54.44	14.00	62.22	8.00	24.42
O5	K-Tetra 11	17.00	59.18	15.00	96.59	9.00	33.53	T-Tetra 12	16.00	54.44	14.00	62.22	8.00	24.42
	K-Tetra 12	16.00	49.81	15.00	96.59	9.00	33.53							
6								T-Tetra 13	17.00	64.09	15.00	73.81	8.00	24.42
nge		**14 - 17**		**13 - 15**		**8 - 9**			**15 - 17**		**13 - 15**		**7 - 8**	
trol		10.68		7.63		6.74			10.36		8.63		6.43	

T - Months after transfer to *ex vitro* environment

Fig 10. Phyllochron values of induced tetraploids and their corresponding diploids



and 17.00d, 15.00 and 17.00d, 14.00 and 17.00d and 15.00 and 17.00d respectively. The phyllochron values for the corresponding diploids of the cultivars Sannachenkadali and Anaikomban were 9.00d each and for those of the cultivars Kunnan and Thattillakunnan were 10.00d each.

Two months after transfer to *ex vitro* environment, the phyllochron values of all the tetraploids ranged between 13.00 and 15.00d. The value for the correspondimg diploids of the cultivars Sannachenkadali, Anaikomban and Kunnan was 7.00d, while that of the cv. Thattillakunnan was 8.00d.

Four months after transfer to *ex vitro* environment, the phyllochron values for the tetraploids derived from the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan ranged between 8.00 and 10.00d, 7.00 and 8.00d, 8.00 and 9.00d and 7.00 and 8.00d respectively. The phyllochron of the corrsponding diploids of all the four cultivars was 6.00d.

4.3.2.2. Leaf LWR (Table 56)

All the induced tetraploids recorded lower average leaf LWR's compared to the corresponding diploids (Table 56).

For the induced tetraploids of the cv. Sannachenkadali, the leaf LW ratios ranged between 1.80 (S-Tetra14) and 1.88 (S-Tetra 7). The corresponding diploid recorded a leaf LWR of 3.45. The tetraploids derived from the cv. Anaikomban recorded leaf LWR's ranging between 1.80 (A-Tetra 8) and 1.91 (A-Tetra 12). The ratio for the corresponding diploid was 3.43. The leaf LWR's for the induced tetraploids of Kunnan ranged between 1.91 (K-Tetra 2) and 1.98 (K-Tetra 9) and that for the corresponding diploid was 3.51. The leaf LWR values for the tetraploids derived from the cv. Thattillakunnan ranged between 1.91 (T-Tetra 6) and 2.01 (T-Tetra 10), while that for the corresponding diploid was 3.61.

Table 56. Leaf LWR of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	Leaf LWR	% increase over control	Name of tetraploid	Leaf LWR	% increase over control	Name of tetraploid	Leaf LWR	% increase over control	Name of tetraploid	Leaf LWR	% increase over control
Colchicine												
C2	S-Tetra1	1.86	-46.09	A-tetra1	1.84	-46.36						
C3	S-Tetra2	1.83	-46.96	A-Tetra2	1.86	-45.77	K-Tetra1	1.94	-44.73	T-Tetra1	1.98	-45.15
	S-Tetra3	1.87	-45.80	A-Tetra3	1.86	-45.77						
C4	S-Tetra4	1.86	-46.09	A-Tetra4	1.89	-44.90	K-Tetra2	1.91	-45.58	T-Tetra2	1.94	-46.26
	S-Tetra5	1.85	-46.38									
C5	S-Tetra6	1.86	-46.09	A-Tetra5	1.84	-46.36	K-Tetra3	1.94	-44.73	T-Tetra3	1.99	-44.88
Oryzalin												
O2	S-Tetra7	1.88	-45.51	A-Tetra6	1.87	-45.48	K-Tetra4	1.92	-45.30	T-Tetra4	1.97	-45.43
		-		A-Tetra7	1.82	-46.94				T-Tetra5	1.98	-45.15
O3	S-Tetra8	1.83	-46.96	A-Tetra8	1.80	-47.52	K-Tetra5	1.93	-45.01	T-Tetra6	1.91	-47.09
	S-Tetra9	1.86	-46.09	A-Tetra9	1.87	-45.48	K-Tetra6	1.96	-44.16	T-Tetra7	1.93	-46.54
	S-Tetra10	1.87	-45.80	A-Tetra10	1.82	-46.94	K-Tetra7	1.94	-44.73	T-Tetra8	1.95	-45.98
	S-Tetra11	1.84	-46.67	A-Tetra11	1.83	-46.65						
O4	S-Tetra12	1.84	-46.67	A-Tetra12	1.91	-44.31	K-Tetra8	1.97	-43.87	T-Tetra9	1.96	-45.71
	S-Tetra13	1.82	-47.25	A-Tetra13	1.84	-46.36	K-Tetra9	1.98	-43.59	T-Tetra10	2.01	-44.32
	S-Tetra14	1.80	-47.83	A-Tetra14	1.89	-44.90	K-Tetra10	1.93	-45.01	T-Tetra11	1.94	-46.26
O5	S-Tetra15	1.85	-46.38	A-Tetra15	1.87	-45.48	K-Tetra11	1.93	-45.01	T-Tetra12	1.95	-45.98
							K-Tetra12	1.94	-44.73			
O6	S-Tetra16	1.86	-46.09	A-Tetra16	1.83	-46.65				T-Tetra13	1.96	-45.71
Range		**1.80 - 1.88**			**1.80 - 1.91**			**1.91 - 1.98**			**1.91 - 2.01**	
Control		3.45			3.43			3.51			3.61	

4.3.2.3. Leaf lamina weight (Table 57)

The leaves of all the induced tetraploids were heavier than those of the corresponding diploids (Table 57).

The weights of leaf lamina of the tetraploids derived from the cv. Sannachenkadali ranged between 46.09g (S-Tetra 4) and 55.36g (S-Tetra 3), while that of the corresponding diploid was 18.54g. The values for the tetraploids derived from the cv. Anaikomban ranged between 46.86g (A-Tetra 6) and 56.43g (S-Tetra 12), while the corresponding diploid recorded a leaf lamina weight of 17.40g. The leaf lamina weights of the tetraploids derived from the cv. Kunnan ranged between 49.31g (K-Tetra 5) and 58.41g (K-Tetra 8). The corresponding diploid recorded a leaf lamina weight of 25.63g. The values for the tetraploids derived from the cv. Thattillakunnan ranged between 47.94g (T-Tetra 12) and 59.41g (T-Tetra 4), while the corresponding diploid of Thattillakunnan recorded a leaf lamina weight of 26.53g.

4.3.2.4. Petiole dry weight percentage (Table 58)

The per cent dry weights of the petioles of all the induced tetraploids were lower than those of the corresponding diploids (Table 58).

The petiole dry weight percentage of the tetraploids derived from the cv. Sannachenkadali ranged between 9.97 (S-Tetra 16) and 12.28 (S-Tetra 1), while that of the corresponding diploid was 14.97. The values for the tetraploids derived from the cv. Anaikomban ranged between 9.76% (A-Tetra 12) and 12.42% (S-Tetra 2), while that of the corresponding diploid was 16.42%. The values for the tetraploids derived from the cv. Kunnan ranged between 9.94% (K-Tetra 3) and 12.76% (K-Tetra 8) and that of the corresponding diploid was 16.54%. The tetraploids derived from the

Table 57. Leaf lamina weight of the *in vitro* derived tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	Weight (g)	% increase over control	Name of tetraploid	Weight (g)	% increase over control	Name of tetraploid	Weight (g)	% increase over control	Name of tetraploid	Weight (g)	% increase over control
Colchicine												
C2	S-Tetra1	46.23	149.35	A-tetra1	53.00	204.60						
C3	S-Tetra2	49.43	166.61	A-Tetra2	48.31	177.64	K-Tetra1	55.41	116.19	T-Tetra1	57.00	114.85
	S-Tetra3	55.36	198.60	A-Tetra3	50.16	188.28						
C4	S-Tetra4	46.09	148.60	A-Tetra4	48.32	177.70	K-Tetra2	56.38	119.98	T-Tetra2	49.64	87.11
	S-Tetra5	48.23	160.14									
C5	S-Tetra6	47.97	158.74	A-Tetra5	48.42	178.28	K-Tetra3	49.62	93.60	T-Tetra3	54.52	105.50
Oryzalin												
O2	S-Tetra7	47.23	154.75	A-Tetra6	46.86	169.31	K-Tetra4	56.96	122.24	T-Tetra4	59.41	123.94
				A-Tetra7	49.97	187.18				T-Tetra5	57.32	116.06
O3	S-Tetra8	46.23	149.35	A-Tetra8	49.17	182.59	K-Tetra5	49.31	92.39	T-Tetra6	51.73	94.99
	S-Tetra9	55.22	197.84	A-Tetra9	48.73	180.06	K-Tetra6	53.98	110.61	T-Tetra7	55.92	110.78
	S-Tetra10	52.78	184.68	A-Tetra10	48.61	179.37	K-Tetra7	56.04	118.65	T-Tetra8	59.32	123.60
	S-Tetra11	49.74	168.28	A-Tetra11	47.64	173.79						
O4	S-Tetra12	54.32	192.99	A-Tetra12	56.43	224.31	K-Tetra8	58.41	127.90	T-Tetra9	50.63	90.84
	S-Tetra13	46.78	152.32	A-Tetra13	49.12	182.30	K-Tetra9	49.72	93.99	T-Tetra10	56.85	114.29
	S-Tetra14	47.63	156.90	A-Tetra14	49.32	183.45	K-Tetra10	50.21	95.90	T-Tetra11	49.44	86.36
O5	S-Tetra15	50.64	173.14	A-Tetra15	54.22	211.61	K-Tetra11	52.31	104.10	T-Tetra12	47.94	80.70
							K-Tetra12	56.41	120.09			
O6	S-Tetra16	51.63	178.48	A-Tetra16	48.41	178.22				T-Tetra13	53.08	100.08
Range		**46.09 - 55.36**			**46.86 - 56.43**			**49.31 - 58.41**			**47.94 - 59.41**	
Control		18.54			17.40			25.63			26.53	

Table 58. Petiole dry weight percentages of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	% dry weight	% increase over control	Name of tetraploid	% dry weight	% increase over control	Name of tetraploid	% dry weight	% increase over control	Name of tetraploid	% dry weight	% increase over control
Colchicine												
C2	S-Tetra1	12.28	-17.97	A-tetra1	11.45	-30.27						
C3	S-Tetra2	10.32	-31.06	A-Tetra2	12.42	-24.36	K-Tetra1	10.76	-34.95	T-Tetra1	11.63	-30.98
	S-Tetra3	11.46	-23.45	A-Tetra3	11.98	-27.04						
C4	S-Tetra4	11.41	-23.78	A-Tetra4	10.56	-35.69	K-Tetra2	11.43	-30.89	T-Tetra2	12.64	-24.99
	S-Tetra5	10.22	-31.73									
C5	S-Tetra6	10.31	-31.13	A-Tetra5	11.61	-29.29	K-Tetra3	9.94	-39.90	T-Tetra3	11.75	-30.27
Oryzalin												
O2	S-Tetra7	11.14	-25.58	A-Tetra6	11.12	-32.28	K-Tetra4	11.42	-30.96	T-Tetra4	11.31	-32.88
				A-Tetra7	10.64	-35.20				T-Tetra5	12.68	-24.75
O3	S-Tetra8	10.24	-31.60	A-Tetra8	11.61	-29.29	K-Tetra5	11.54	-30.23	T-Tetra6	11.76	-30.21
	S-Tetra9	11.09	-25.92	A-Tetra9	10.32	-37.15	K-Tetra6	10.75	-35.01	T-Tetra7	10.14	-39.82
	S-Tetra10	10.36	-30.79	A-Tetra10	11.73	-28.56	K-Tetra7	11.76	-28.90	T-Tetra8	9.64	-42.79
	S-Tetra11	12.16	-18.77	A-Tetra11	10.24	-37.64						
O4	S-Tetra12	11.75	-21.51	A-Tetra12	9.76	-40.56	K-Tetra8	12.76	-22.85	T-Tetra9	11.33	-32.76
	S-Tetra13	10.14	-32.26	A-Tetra13	11.74	-28.50	K-Tetra9	12.31	-25.57	T-Tetra10	12.21	-27.54
	S-Tetra14	10.72	-28.39	A-Tetra14	11.53	-29.78	K-Tetra10	11.75	-28.96	T-Tetra11	10.98	-34.84
O5	S-Tetra15	11.31	-24.45	A-Tetra15	11.18	-31.91	K-Tetra11	11.32	-31.56	T-Tetra12	11.76	-30.21
							K-Tetra12	12.65	-23.52			
O6	S-Tetra16	9.97	-33.40	A-Tetra16	10.42	-36.54				T-Tetra13	10.76	-36.14
Range		**9.97 - 12.28**			**9.76 - 12.42**			**9.94 - 12.76**			**9.64 - 12.68**	
Control		14.97			16.42			16.54			16.85	

cv. Thattillakunnan recorded petiole dry weight percentages ranging between 9.64 (T-Tetra 8) and 12.68 (T-Tetra 5)and the corresponding diploid recorded a value of 16.85%.

4.3.2.5. **Photosynthetic efficiency** (Table 59)

All the induced tetraploids recorded higher photosynthetic efficiencies than the corresponding diploids (Table 59).

The photosynthetic efficiencies of the tetraploids derived from the cv. Sannachenkadali ranged between 0.584 (S-Tetra 3) and 0.621 (S-Tetra 2). The photosynthetic efficiency of the corresponding diploid was 0.510.

The values for the tetraploids derived from the cv. Anaikomban ranged between 0.591 (A-Tetra 13) and 0.621 (S-Tetra 2), while that for the corresponding diploid was 0.508. The photosynthetic efficiencies of the tetraploids derived from the cv. Kunnan ranged between 0.603 (K-Tetra 1) and 0.621 (K-Tetra 10), while that of the corresponding diploid was 0.563. The tetraploids derived from the cv. Thattillakunnan recorded photosynthetic efficiencies ranging between 0.604 (T-Tetra3) and 0.618 (T-Tetra 6) and the value for the corresponding diploid was 0.546.

4.3.2.6. **Crop growth rate** (Tables 60a and b; Fig. 11)

The crop growth rates of the tetraploids were lower than those of the respective diploid plants initially (when observed 2 months after transfer to *ex vitro* environment). On the other hand, at 4 months after transfer to *ex vitro* environment the crop growth rates of the induced tetraploids hed surpassed those of their respective diploids (Tables 60a and b).

The crop growth rates two months after transfer ranged between 0.235-0.283cm day^{-1}, 0.253-0.339cm day^{-1}, 0.251-0.308 cm day^{-1} and 0.250-0.338 cm day^{-1} for the induced tetraploids of

Table 59. Photosynthetic efficiencies of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	Photosyntetic efficiency (Quantum units)	% increase over control	Name of tetraploid	Photosyntetic efficiency (Quantum units)	% increase over control	Name of tetraploid	Photosyntetic efficiency (Quantum units)	% increase over control	Name of tetraploid	Photosyntetic efficiency (Quantum units)	% increase over control
Colchicine												
C2	S-Tetra1	0.597	17.06	A-tetra1	0.602	18.50						
C3	S-Tetra2	0.621	21.76	A-Tetra2	0.621	22.24	K-Tetra1	0.603	7.10	T-Tetra1	0.608	11.36
	S-Tetra3	0.584	14.51	A-Tetra3	0.595	17.13						
C4	S-Tetra4	0.606	18.82	A-Tetra4	0.598	17.72	K-Tetra2	0.612	8.70	T-Tetra2	0.606	10.99
	S-Tetra5	0.612	20.00									
C5	S-Tetra6	0.606	18.82	A-Tetra5	0.598	17.72	K-Tetra3	0.608	7.99	T-Tetra3	0.604	10.62
Oryzalin												
O2	S-Tetra7	0.586	14.90	A-Tetra6	0.594	16.93	K-Tetra4	0.611	8.53	T-Tetra4	0.612	12.09
				A-Tetra7	0.602	18.50				T-Tetra5	0.613	12.27
O3	S-Tetra8	0.601	17.84	A-Tetra8	0.601	18.31	K-Tetra5	0.612	8.70	T-Tetra6	0.618	13.19
	S-Tetra9	0.606	18.82	A-Tetra9	0.594	16.93	K-Tetra6	0.609	8.17	T-Tetra7	0.613	12.27
	S-Tetra10	0.598	17.25	A-Tetra10	0.597	17.52	K-Tetra7	0.614	9.06	T-Tetra8	0.606	10.99
	S-Tetra11	0.602	18.04	A-Tetra11	0.610	20.08						
O4	S-Tetra12	0.597	17.06	A-Tetra12	0.612	20.47	K-Tetra8	0.608	7.99	T-Tetra9	0.616	12.82
	S-Tetra13	0.601	17.84	A-Tetra13	0.591	16.34	K-Tetra9	0.613	8.88	T-Tetra10	0.605	10.81
	S-Tetra14	0.604	18.43	A-Tetra14	0.606	19.29	K-Tetra10	0.621	10.30	T-Tetra11	0.609	11.54
O5	S-Tetra15	0.597	17.06	A-Tetra15	0.602	18.50	K-Tetra11	0.619	9.95	T-Tetra12	0.609	11.54
							K-Tetra12	0.620	10.12			
O6	S-Tetra16	0.603	18.24	A-Tetra16	0.613	20.67				T-Tetra13	0.618	13.19
Range		**0.584 - 0.621**			**0.591 - 0.621**			**0.603 - 0.621**			**0.604 - 0.618**	
Control		0.510			0.508			0.563			0.546	

Table 60(a). Crop growth rates (cm day^{-1}) of the *in vitro* induced tetraploids derived from the cultivars Sannachenkadali and Anaikomban

Treatment	Sannachenkadali					Anaikomban				
	Name of tetraploid	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine										
C2	S-Tetra 1	0.271	-29.32	0.550	25.96	A-Tetra 1	0.293	-19.55	0.548	26.78
C3	S-Tetra 2	0.256	-33.23	0.543	24.43	A-Tetra 2	0.270	-25.73	0.551	27.34
	S-Tetra 3	0.283	-26.24	0.551	26.13	A-Tetra 3	0.299	-17.99	0.560	29.42
C4	S-Tetra 4	0.279	-27.37	0.540	23.78	A-Tetra 4	0.283	-22.39	0.551	27.46
	S-Tetra 5	0.256	-33.28	0.532	21.89					
C5	S-Tetra 6	0.266	-30.63	0.541	23.92	A-Tetra 5	0.253	-30.40	0.538	24.32
Oryzalin										
O2	S-Tetra 7	0.269	-29.89	0.550	25.98	A-Tetra 6	0.283	-22.39	0.576	33.06
						A-Tetra 7	0.292	-19.92	0.578	33.64
O3	S-Tetra 8	0.274	-28.67	0.543	24.37	A-Tetra 8	0.268	-26.37	0.551	27.48
	S-Tetra 9	0.249	-35.06	0.550	25.88	A-Tetra 9	0.277	-23.81	0.550	27.19
	S-Tetra 10	0.235	-38.75	0.542	24.15	A-Tetra 10	0.277	-23.95	0.543	25.43
	S-Tetra 11	0.238	-38.05	0.540	23.69	A-Tetra 11	0.270	-25.92	0.534	23.51
O4	S-Tetra 12	0.238	-38.01	0.543	24.43	A-Tetra 12	0.296	-18.64	0.546	26.22
	S-Tetra 13	0.264	-31.15	0.557	27.66	A-Tetra 13	0.298	-18.09	0.546	26.26
	S-Tetra 14	0.256	-33.32	0.547	25.18	A-Tetra 14	0.329	-9.62	0.551	27.34
O5	S-Tetra 15	0.250	-34.75	0.552	26.34	A-Tetra 15	0.339	-6.87	0.560	29.42
O6	S-Tetra 16	0.253	-34.14	0.563	28.96	A-Tetra 16	0.320	-12.18	0.559	29.15
Range		**0.235 - 0.283**		**0.532 - 0.563**			**0.253 - 0.339**		**0.534 - 0.578**	
Control		0.384		0.437			0.364		0.433	

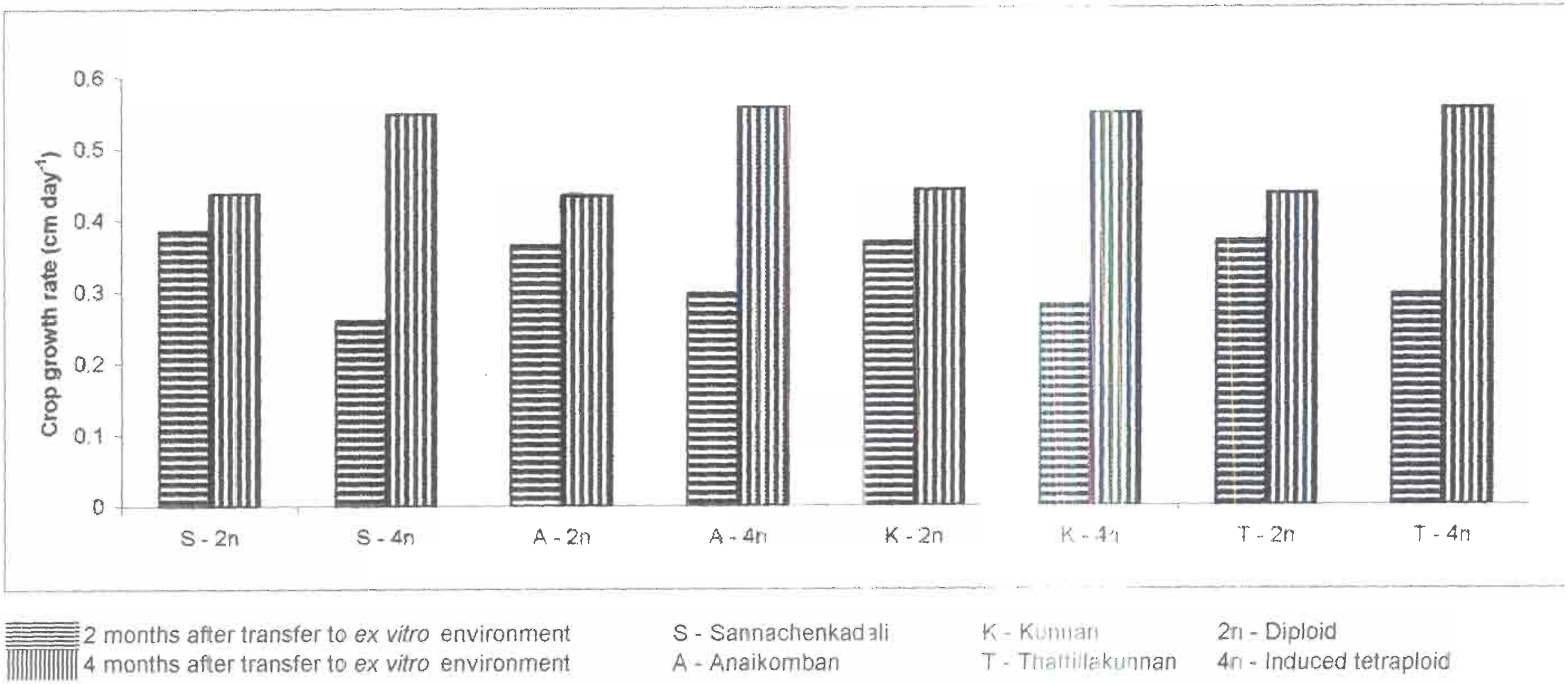
* MAT - Months after transfer to *ex vitro* environment

Table 60(b). ***Crop growth rates*** **(cm day^{-1})** ***of the in vitro*** **induced tetraploids derived from the cultivars Kunnan and Thattillakunnan**

Treatment	Kunnan					Thattillakunnan				
	Name of tetraploid	2 MAT*	% difference over control	4 MAT	% difference over control	Name of tetraploid	2 MAT	% difference over control	4 MAT	% difference over control
Colchicine										
C3	K-Tetra 1	0.251	-31.70	0.524	18.95	T-Tetra 1	0.250	-32.10	0.542	24.75
C4	K-Tetra 2	0.266	-27.62	0.526	19.25	T-Tetra 2	0.262	-29.02	0.547	25.86
C5	K-Tetra 3	0.273	-25.85	0.547	24.04	T-Tetra 3	0.282	-23.60	0.539	24.10
Oryzalin										
O2	K-Tetra 4	0.269	-26.80	0.571	29.58	T-Tetra 4	0.325	-11.89	0.568	30.76
						T-Tetra 5	0.277	-25.00	0.560	28.93
O3	K-Tetra 5	0.300	-18.50	0.566	28.54	T-Tetra 6	0.255	-30.83	0.567	30.62
	K-Tetra 6	0.252	-31.43	0.559	26.93	T-Tetra 7	0.257	-30.38	0.550	26.61
	K-Tetra 7	0.283	-22.95	0.566	28.46	T-Tetra 8	0.282	-23.60	0.550	26.65
O4	K-Tetra 8	0.278	-24.35	0.564	28.08	T-Tetra 9	0.338	-8.32	0.562	29.32
	K-Tetra 9	0.278	-24.40	0.539	22.26	T-Tetra 10	0.300	-18.76	0.541	24.65
	K-Tetra 10	0.308	-16.19	0.541	22.83	T-Tetra 11	0.318	-13.79	0.543	25.10
O5	K-Tetra 11	0.286	-22.31	0.547	24.19	T-Tetra 12	0.332	-9.90	0.558	28.45
	K-Tetra 12	0.303	-17.60	0.550	24.74					
O6						T-Tetra 13	0.306	-16.91	0.545	25.38
Range		**0.251 - 0.308**		**0.524 - 0.571**			**0.250 - 0.338**		**0.539 - 0.567**	
Control		0.368		0.441			0.369		0.434	

* MAT - Months after transfer to *ex vitro* environment

Fig 11. Crop growth rates of induced tetraploids and their corresponding diploids



the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively, while those of their respective diploids were 0.384 cm day^{-1} , 0.364 cm day^{-1}, 0.368 cm day^{-1} and 0.369 cm day^{-1} respectively.

Four months after transfer of the plants to *ex vitro* environment, the crop growth rates of the induced tetraploids of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively ranged between 0.532-0.563cm day^{-1}, 0.534-0.578cm day^{-1}, 0.524-0.571cm day^{-1} and 0.539-0.567cm day^{-1}, while those of the corresponding diploids were 0.437 cm day^{-1} 0.433 cm day^{-1} 0.441 cm day^{-1} and 0.434 cm day^{-1} for the cultivars Sannachenkadali ,Anaikomban, Kunnan and Thattillakunnan respectively.

4.3.3. Effect of tetraploidy on biochemical parameters

4.3.3.1. Chlorophyll content (Table 61)

The chlorophyll contents of the induced tetraploids were higher than those of the corresponding diploid (Table 61).

The chlorophyll contents of the tetraploids derived from the cv. Sannachenkadali ranged between 2.82mggl^{-1} (S-Tetra 5) and 3.14 mggl^{-1} (S-Tetra 13), while that of the corresponding diploid was 2.14mggl^{-1}. The chlorophyll contents of the tetraploids derived from the cv. Anaikomban ranged between 2.79mggl^{-1} (A-Tetra 4) and 3.06mggl^{-1} (S-Tetra 1) and that of the corresponding diploid was 2.11 mggl^{-1}. The tetraploids derived from the cv. Kunnan had chlorophyll contents ranging between 2.94mggl^{-1} (K-Tetra 1) and 3.21mggl^{-1} (K-Tetra 12), while the corresponding diploid recorded 2.24 mggl^{-1} of chlorophyll content.. The chlorophyll contents of the tetraploids derived from the cv. Thattillakunnan ranged between 2.91mggl^{-1} (T-Tetra 3) and 3.18mggl^{-1} (T-Tetra 8), and that of the corresponding diploid was 2.19 mggl^{-1}.

Table 61. Chlorophyll contents of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	Chlorophyll (mgg^{-1})	% increase over control	Name of tetraploid	Chlorophyll (mgg^{-1})	% increase over control	Name of tetraploid	Chlorophyll (mgg^{-1})	% increase over control	Name of tetraploid	Chlorophyll (mgg^{-1})	% increase over control
Colchicine												
C2	S-Tetra1	2.89	35.05	A-tetra1	3.06	45.02						
C3	S-Tetra2	2.93	36.92	A-Tetra2	2.92	38.39	K-Tetra1	2.94	31.25	T-Tetra1	2.98	36.07
	S-Tetra3	2.96	38.32	A-Tetra3	2.98	41.23						
C4	S-Tetra4	3.04	42.06	A-Tetra4	2.79	32.23	K-Tetra2	3.04	35.71	T-Tetra2	2.98	36.07
	S-Tetra5	2.82	31.78									
C5	S-Tetra6	2.86	33.64	A-Tetra5	2.81	33.18	K-Tetra3	3.11	38.84	T-Tetra3	2.91	32.88
Oryzalin												
O2	S-Tetra7	2.98	39.25	A-Tetra6	2.86	35.55	K-Tetra4	2.99	33.48	T-Tetra4	3.09	41.10
				A-Tetra7	3.04	44.08				T-Tetra5	3.03	38.36
O3	S-Tetra8	2.93	36.92	A-Tetra8	3.04	44.08	K-Tetra5	2.96	32.14	T-Tetra6	3.14	43.38
	S-Tetra9	2.97	38.79	A-Tetra9	2.96	40.28	K-Tetra6	3.08	37.50	T-Tetra7	2.99	36.53
	S-Tetra10	2.94	37.38	A-Tetra10	2.88	36.49	K-Tetra7	3.01	34.38	T-Tetra8	3.18	45.21
	S-Tetra11	2.92	36.45	A-Tetra11	2.93	38.86						
O4	S-Tetra12	2.98	39.25	A-Tetra12	2.93	38.86	K-Tetra8	3.12	39.29	T-Tetra9	3.13	42.92
	S-Tetra13	3.14	46.73	A-Tetra13	3.04	44.08	K-Tetra9	3.10	38.39	T-Tetra10	3.08	40.64
	S-Tetra14	2.93	36.92	A-Tetra14	2.98	41.23	K-Tetra10	3.09	37.95	T-Tetra11	2.99	36.53
O5	S-Tetra15	2.97	38.79	A-Tetra15	2.86	35.55	K-Tetra11	2.98	33.04	T-Tetra12	3.08	40.64
							K-Tetra12	3.21	43.30			
O6	S-Tetra16	2.90	35.51	A-Tetra16	2.83	34.12				T-Tetra13	3.14	43.38
Range		**2.82 - 3.14**			**2.79 - 3.06**			**2.94 - 3.21**			**2.91 - 3.18**	
Control		2.14			2.11			2.24			2.19	

4.3.3.2. **Anthocyanin content** (Table 62; Plate 39)

The data (Table 62, Plate 39) revealed the presence of higher anthocyanin contents in the leaves and petioles of the tetraploids derived from the cv. Sannachenkadali, compared to the corresponding diploids.

The leaf anthocyanin contents of the tetraploids ranged between 229.71 (Tetra 10) and 249.24 mg/100g (S-Tetra 6) and that for the corresponding diploid was 136.43 mg/100g. The petiolar anthocyanin contents of the tetraploids ranged between 254.18 (S-Tetra 12) and 271.49 mg/100g (S-Tetra 7). The corresponding diploid recorded an anthocyanin content of 173.14mg/100g.

4.3.4. Effect of tetraploidy on cytogenetic parameters

4.3.4.1. **Interphase nuclear volume** (Table 63; ~~Figs~~ Plates 40 and 41)

All the induced tetraploids recorded larger interphase nuclear volumes compared to the corresponding diploid (Table 63, Plates 40 and 41).

The INV's of the tetraploids derived from the cv. Sannachenkadali ranged between $1109.28\mu^3$ (S-Tetra 16) and $1416.31\mu^3$ (S-Tetra 3). The corresponding diploid had an INV of $426.38\mu^3$. The INV's of the tetraploids derived from the cv. Anaikomban ranged between $1123.33\mu^3$ (A-Tetra 11) and $1386.14\mu^3$ (S-Tetra 6), while that of the corresponding diploid was $434.29\mu^3$. The tetraploids derived from the cv. Kunnan had INV's ranging between $1129.31\mu^3$ (K-Tetra 11) and $1420.32\mu^3$ (K-Tetra 2) and the corresponding diploid had an INV of $442.14\mu^3$. The INV's of the tetraploids derived from the cv. Thattillakunnan ranged between $1098.32\mu^3$ (T-Tetra 2) and $1364.31\mu^3$ (T-Tetra 8), while that of the corresponding diploid was $412.69\mu^3$.

Table 62. Anthocyanin contents of the *in vitro* induced tetraploids derived from the cv. Sannachenkadali

Treatment	Name of tetraploid	Anthocyanin content in leaf lamina (mg $100g^{-1}$)	% difference over control	Anthocyanin content in leaf petiole (mg $100g^{-1}$)	% difference over control
Colchicine					
C2	S-Tetra1	249.08	82.57	261.22	50.87
C3	S-Tetra2	236.26	73.17	270.23	56.08
	S-Tetra3	239.14	75.28	268.78	55.24
C4	S-Tetra4	242.12	77.47	262.21	51.44
	S-Tetra5	231.26	69.51	258.09	49.06
C5	S-Tetra6	249.24	82.69	263.33	52.09
Oryzalin					
O2	S-Tetra7	246.16	80.43	271.49	56.80
O3	S-Tetra8	234.42	71.82	264.31	52.66
	S-Tetra9	231.30	69.54	258.41	49.25
	S-Tetra10	229.71	68.37	264.36	52.69
	S-Tetra11	248.28	81.98	266.22	53.76
O4	S-Tetra12	241.64	77.12	254.18	46.81
	S-Tetra13	240.26	76.10	262.02	51.33
	S-Tetra14	243.20	78.26	269.21	55.49
O5	S-Tetra15	238.81	75.04	264.56	52.80
O6	S-Tetra16	234.14	71.62	263.23	52.03
Range		**229.71 - 249.24**		**254.18 - 271.49**	
Control		136.43		173.14	

Table 63. Interphase nuclear volume of the *in vitro* induced tetraploids

Treatment	Sannachenkadali			Anaikomban			Kunnan			Thattillakunnan		
	Name of tetraploid	INV(μ^3)	% increase over control	Name of tetraploid	INV(μ^3)	% increase over control	Name of tetraploid	INV(μ^3)	% increase over control	Name of tetraploid	INV(μ^3)	% increase over control
Colchicine												
C2	S-Tetra1	1278.14	199.77	A-tetra1	1261.22	190.41						
C3	S-Tetra2	1296.28	204.02	A-Tetra2	1138.43	162.14	K-Tetra1	1176.23	166.03	T-Tetra1	1236.23	199.55
	S-Tetra3	1416.31	232.17	A-Tetra3	1341.61	208.92						
C4	S-Tetra4	1208.16	183.35	A-Tetra4	1216.20	180.04	K-Tetra2	1420.32	221.24	T-Tetra2	1098.32	166.14
	S-Tetra5	1124.73	163.79									
C5	S-Tetra6	1314.28	208.24	A-Tetra5	1273.33	193.20	K-Tetra3	1236.11	179.57	T-Tetra3	1102.31	167.10
Oryzalin												
O2	S-Tetra7	1326.42	211.09	A-Tetra6	1386.14	219.17	K-Tetra4	1268.56	186.91	T-Tetra4	1312.58	218.05
				A-Tetra7	1157.35	166.49				T-Tetra5	1145.26	177.51
O3	S-Tetra8	1193.31	179.87	A-Tetra8	1239.46	185.40	K-Tetra5	1329.43	200.68	T-Tetra6	1196.38	189.90
	S-Tetra9	1129.60	164.93	A-Tetra9	1163.48	167.90	K-Tetra6	1262.64	185.57	T-Tetra7	1121.41	171.73
	S-Tetra10	1239.49	190.70	A-Tetra10	1198.54	175.98	K-Tetra7	1293.39	192.53	T-Tetra8	1364.31	230.59
	S-Tetra11	1386.50	225.18	A-Tetra11	1123.33	158.66						
O4	S-Tetra12	1169.46	174.28	A-Tetra12	1146.61	164.02	K-Tetra8	1139.73	157.78	T-Tetra9	1293.27	213.38
	S-Tetra13	1123.45	163.49	A-Tetra13	1308.51	201.30	K-Tetra9	1216.71	175.19	T-Tetra10	1321.71	220.27
	S-Tetra14	1261.52	195.87	A-Tetra14	1265.50	191.40	K-Tetra10	1231.67	178.57	T-Tetra11	1106.22	168.05
O5	S-Tetra15	1326.35	211.07	A-Tetra15	1216.17	180.04	K-Tetra11	1129.31	155.42	T-Tetra12	1126.34	172.93
							K-Tetra12	1341.26	203.36			
O6	S-Tetra16	1109.28	160.16	A-Tetra16	1238.30	185.13				T-Tetra13	1230.16	198.08
Range		**1109.28 - 1416.31**			**1123.33 - 1386.14**			**1129.31 - 1420.32**			**1098.32 - 1364.31**	
Control		426.38			434.29			442.14			412.69	

DISCUSSION

CHAPTER V
DISCUSSION

Breeding of most commercially acceptable bananas is complicated by their parthenocarpic nature and triploidy and hence, non-conventional breeding activities are being investigated in recent times, to complement and support the conventional breeding activities (Swennen and Vuylsteke, 1988). One of the methods to obtain synthetic triploids would be to induce chromosome doubling of promising diploids and then crossing them with improved diploids (Novak, 1992).

The present investigations carried out at the Tissue Culture Laboratory of the Department of Fruit Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore aimed at optimising a method for inducing tetraploids under *in vitro* conditions from diploid starting material, involving anti-mitotic agents that induce chromosome doubling. The diploid cultivars involved were Sannachenkadali (AA), Anaikomban (AA), Kunnan (AB) and Thattillakunnan (AB). The cv. Sannachenkadali was chosen for its resistance to leaf spot disease (*Mycosphaerella* spp.), Anaikomban for its resistance to burrowing nematode (*Radopholus similis*), Kunnan for its tolerance to Panama disease (*Fusarium oxysporum* f.sp. *cubense*), leaf spot and burrowing nematode and Thattillakunnan for its tolerance to burrowing nematode The anti-mitotic agents employed were colchicine and oryzalin. The results of the investigations are discussed hereunder.

IMPACT OF ANTI-MITOTIC AGENTS

It is a well-established fact that anti-mitotic substances are associated with an initial growth retardation. In agreement with such a fact, in the present investigations, the *in vitro* regeneration rates of the anti-mitotic agent treated shoot tip cultures were very much retarded. Such a retarded growth response was reflected in terms of delay in regeneration, reduced multiple shoot regeneration rates, regeneration of smaller microshoots with lower fresh weights and reduced response to rhizogenesis.

Delayed response to *in vitro* regeneration

Shoot tips treated with colchicine and oryzalin took considerably longer periods than the untreated control to produce the first visual positive response to *in vitro* regeneration, *viz.*, greening of the explant followed by emergence of the first leaf. The time required for greening of shoot tips increased with increase in concentration of anti-mitotic agent and duration of the treatment. Further, extension in the time required for shoot tip greening caused by the increasing duration of treatment was higher with colchicine than with oryzalin. This can be explained on the basis of the fact that colchicine is associated with the existence of a persistent spindle inhibition (Bowen and Wilson, 1954). Accordingly, the shorter treatment duration, *viz.*, 12h would have delayed the response of the shoot tips to *in vitro* regeneration to a certain extent. Extending the duration of treatment to 24h and 36h would have triggered the persisting spindle inhibition activity resulting in a much delayed response.

In the present investigations, it was observed that treatments involving prolonged exposure (6d) of cultures to oryzalin were not as deleterious as those involving prolonged exposure to colchicine (24h and 36h). Opinion of earlier workers (Cleary and Hardham, 1987) that variations existed among plant species in the treatment times required to achieve microtubule depolymerization by oryzalin in a majority of dividing cells, and that most monocotyledons required comparatively lower exposure times than dicotyledons provides explanation to such an observation. *Musa* spp. being a monocotyledon, might have required lower exposure times to oryzalin to achieve microtubular depolymerization in a majority of cells. After the maximum microtubule depolymerization had been achieved, further exposure to oryzalin would not have had any effect on the cultures, unlike the case with colchicine which has been proved to be associated with a persistent spindle inhibition. Such an observation is further corroborated by the view of Strachan and Hess (1983) as per which, an equilibrium was reached once the denaturation of tubulin by oryzalin had commenced, further treatment not causing any impact.

Delay caused by colchicine and oryzalin in the greening of shoot tip, emergence of the first leaf and initiation of roots was relatively higher in the cultivars Kunnan (AB) and Thattillakunnan (AB) than in Sannachenkadali (AA) and Anaikomban (AA). This indicated the fact that response of the cultivars to anti-mitotic agents varied with the genomic constitution.

Reduced *in vitro* regeneration rates

Colchicine- and oryzalin-treated cultures recorded lower multiple shoot regeneration rates (Fig. 8) and regenerated shorter microshoots with lower fresh weights, compared to the untreated control. The response to multiple shoot regeneration in the primary culture and the subsequent three vegatative cycles exhibited a negative correlation with the concentration of colchicine and oryzalin and the duration of treatment.

Inhibited synthesis and activity of auxins (Smith and Kersten, 1942; Gaur and Notani, 1960; Rajput and Qureshi, 1973, Miura *et al.*, 1974), genetic injury in dividing cells (Caldecott, 1961), deficiency or inhibition of some physiological pre-requisite to cell division (Stein and Sparrow, 1963), inhibition of the specific activity of certain enzymes (Endo, 1967) and inhibition of DNA synthesis (Mikaelson *et al.*, 1968) might be attributed as some of the probable causes for the inhibited rates of shoot regeneration. Such an observation is in agreement with that of van Duren *et al.* (1996) who reported that the effect of colchicine and oryzalin treatments on *in vitro* cultures of banana was reflected in a lower shoot regeneration rate.

Similar to the case with the time taken for responding to *in vitro* regeneration, the cultivars varied in the degree of response to multiple shoot regeneration. At all stages (starting from the primary culture phase to the third vegetative cycle), the reduction in the rates of response to multiple shoot regeneration and the number of microshoots regenerated per culture was comparatively higher in the cultivars Kunnan (AB) and Thattillakunnan (AB) than in the cultivars Sannachenkadali (AA) and Anaikomban (AA). This might be attributed to the general tendency of *Musa* cultivars with the 'B' genome in their genomic constitution to have a higher degree of apical dominance, resulting in comparatively lower response to multiple shoot regeneration than pure 'acuminata' types.

Growth stimulating action of oryzalin

It was interesting to observe that contrary to the expectation that both the anti-mitotic agents would cause a decline in the number of microshoots regenerated, oryzalin at lower concentrations resulted in regeneration of higher number of microshoots per culture, the number being higher than that of the untreated control. Colchicine, as expected, had a negative effect on the number of multiple shoots regenerated. The basis behind such a tendency can be explained by the fact that a few of the dinitroaniline herbicides are growth stimulating at lower concentrations, having lower growth inhibiting effects (Hansen and

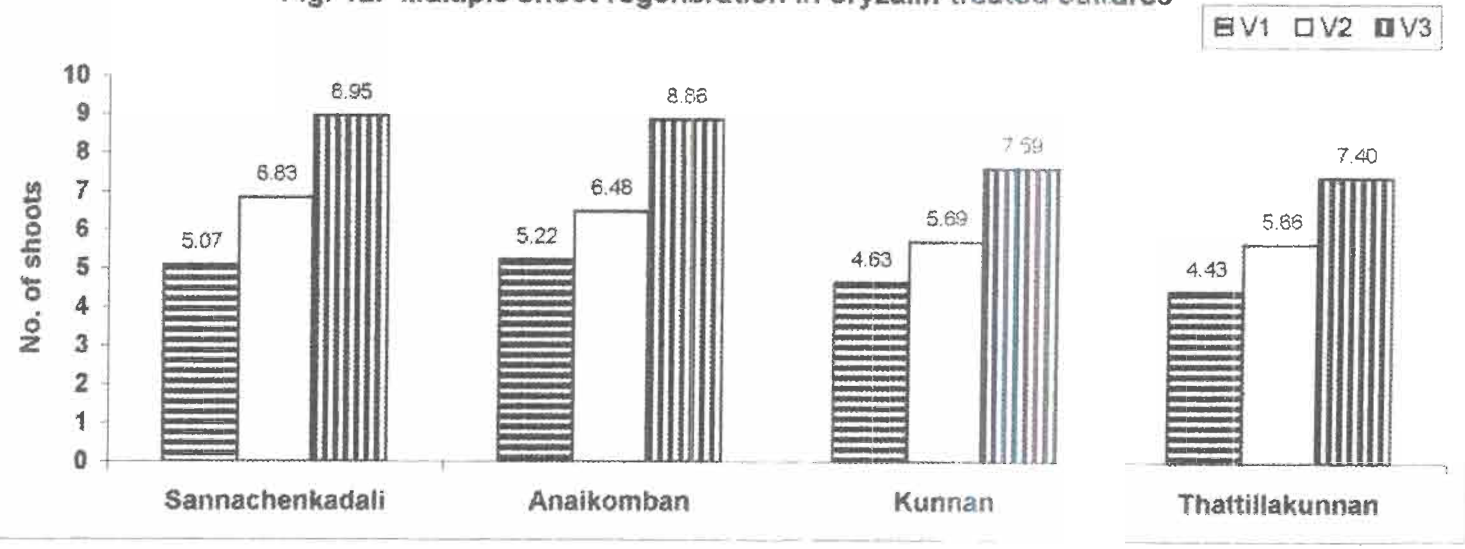
Andersen, 1996). Moreover, involvement of mechanisms other than blockage of microtubules are believed to have caused growth stimulation at lower concentration of these compounds. Such a growth stimulation at low concentrations of certain toxic chemical compounds is not uncommon (Brain and Cousens, 1989). Stronger corroboration of this fact comes from van Duren *et al.* (1996) who observed that meristem cultures of *M. acuminata* clone SH-3362 treated with lower oryzalin concentration (15μM) had a tendency to produce more shoots.

The growth stimulation effect associated with oryzalin was higher in the cultivars Sannachenkadali and Anaikomban than in Kunnan and Thattillakunnan (Fig. 12). This resulted in regeneration of higher number of shoots from the cultures of the cultivars Sannachenkadali and Anaikomban than those of Kunnan and Thattillakunnan in all the three vegetative cycles. Such a tendency might again be attributed to the lower degree of apical dominance associated with pure 'acuminata' types, which when subjected to the growth stimulative action of lower oryzalin concentrations might have led to regeneration of higher number of microshoots.

Reduced response to rhizogenesis

The colchicine- and oryzalin-treated cultures expressed a similar trend of response to the factors related to rhizogenesis namely, the rate of response to rhizogenesis, the time taken for initiation of roots, the number of roots produced per plantlet and the length of roots. All these characters were negatively correlated with the concentration of the anti-mitotic agent and the treatment duration. Further, the negative effect associated with longer treatment duration was higher with colchicine than with oryzalin, similar to the case with shoot regeneration. Inhibition of the

Fig. 12. Multiple shoot regeneration in oryzalin-treated cultures



V1 - I vegetative cycle V2 - II vegetative cycle V3 - III vegetative cycle synthesis and activity of auxins (Smith and Kersten, 1942; Gaur and Notani, 1960) is the possible cause for the retarded response to rhizogenesis.

PLOIDY-RELATED CONSEQUENCES OF INDUCED POLYPLOIDY

Induction of tetraploidy

In the present investigations, the treatment involving the lowest concentration of colchicine did not induce tetraploidy, while those involving higher concentrations induced varying rates of tetraploidy. This can be explained by the fact that destruction of some link in the spindle-forming reaction is caused by colchicine only when a certain concentration is reached (Nebel and Ruttle, 1938). Further corroboration of this observation comes from the opinion of Vakili (1967) who observed that the higher the concentration of colchicine, the greater the percentage of tetraploids formed in the seedlings of *M. acuminata.*

With respect to the rates of tetraploidy induced, a similar trend was followed by colchicine and oryzalin. In both the cases, the rates of tetraploidy induced increased with increase in the concentration of the anti-mitotic agent, the duration of treatment remaining unchanged. However, increasing the duration of treatment caused a variation in the effects of the two anti-mitotic agents on ploidy. Prolonged colchicine treatments (24h and 36h) caused a decline in the rates of tetraploidy induced, with a corresponding increase in the rates of 'other' ploids. On the other hand, prolonged oryzalin treatment (6d), though caused a decline in the rates of tetraploidy induced, was not associated with increase in the rates of 'other' ploids, unlike the case with colchicine. These observations are attributable to the persisting spindle inhibition associated with colchicine (Bowen and Wilson, 1954) and the attaining of equilibrium once denaturation of tubulin by oryzalin had commenced (Strachan and Hess, 1983). The persisting spindle inhibition activity of colchicine might have led to higher degree of alterations in the ploidy status leading to the 'other' ploids. In the case of oryzalin, since equilibrium is reached once tubulin denaturation commences, prolonged treatment would not have caused further ploidy alterations, resulting in lower rates of 'other' ploids.

Superiority of oryzalin over colchicine

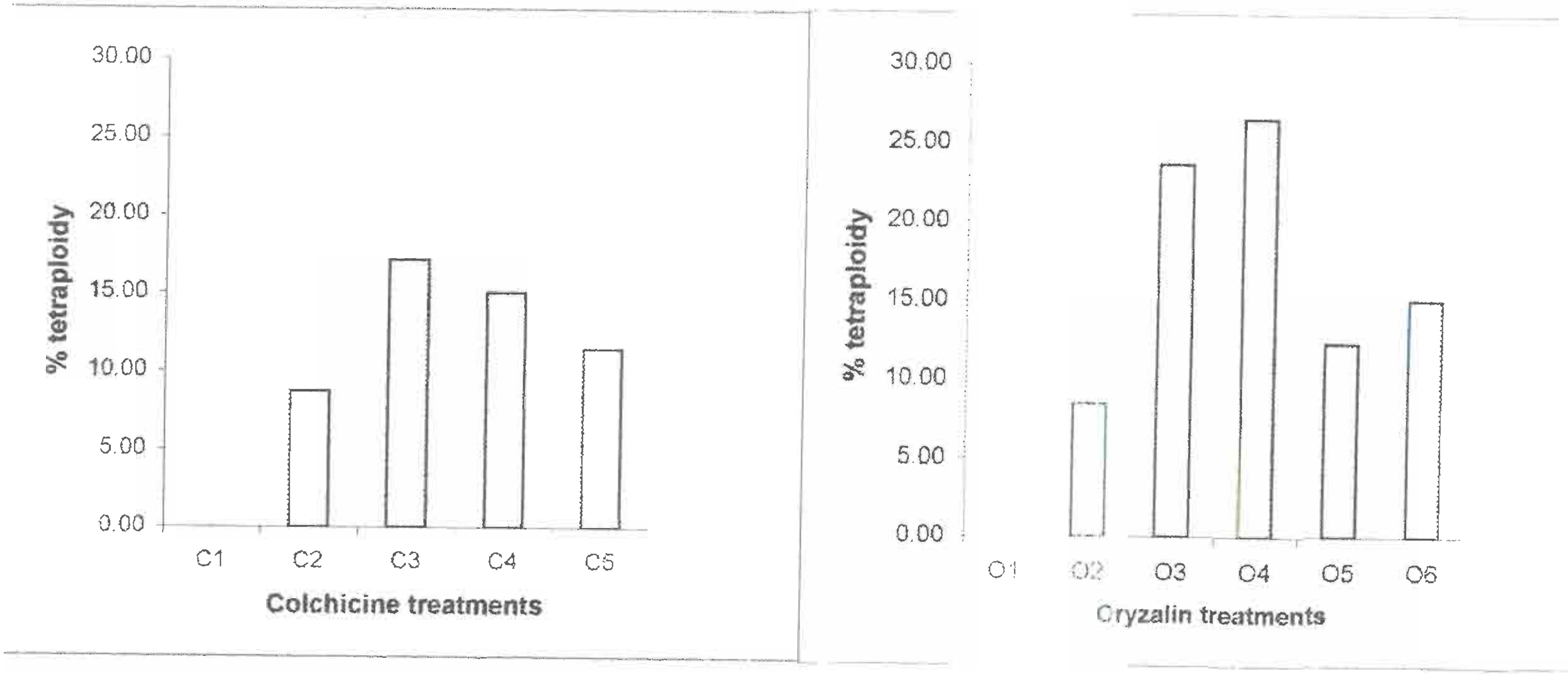
Variations existed in the chromosome doubling capacities of colchicine and oryzalin (Fig.13).

Comparison of the chromosome doubling capacities of colchicine and oryzalin

Conc. of colchicine(μM)	Per cent tetraploidy induced	Conc. of oryzalin(μM)	Per cent tetraploidy induced	Increase in conc. of colchicine over that of oryzalin
5000	8.70	20	8.43	250 times
2500	15.03	20	15.03	125 times

The chromosome doubling capacity of colchicine was equal to that of oryzalin at 125-200 times higher concentrations. The highest rate of tetraploidy achieved with the oryzalin treatments was 26.53 per cent, the concentration which induced this being 40μM. On the other hand, among the colchicine treatments, the highest rate of tetraploidy, *viz.*, 17.09 per cent was induced by the treatment which involved 7.5mM of colchicine. Thus it has been observed that the chromosome doubling efficiency of colchicine at a concentration 187.5 times higher was lower than that of oryzalin. The requirement of oryzalin at lower concentrations to induce effects similar to those of colchicine at much higher concentrations, might be attributed to the higher affinity of

Fig. 13. Comparison of the chromosome doubling capacities of colchicine and oryzalin



C1 - 2.5mM colchicine for 12h
C2 - 5.0mM colchicine for 12h
C3 - 7.5mM colchicine for 12h
C4 - 2.5mM colchicine for 24h
C5 - 2.5mM colchicine for 36h

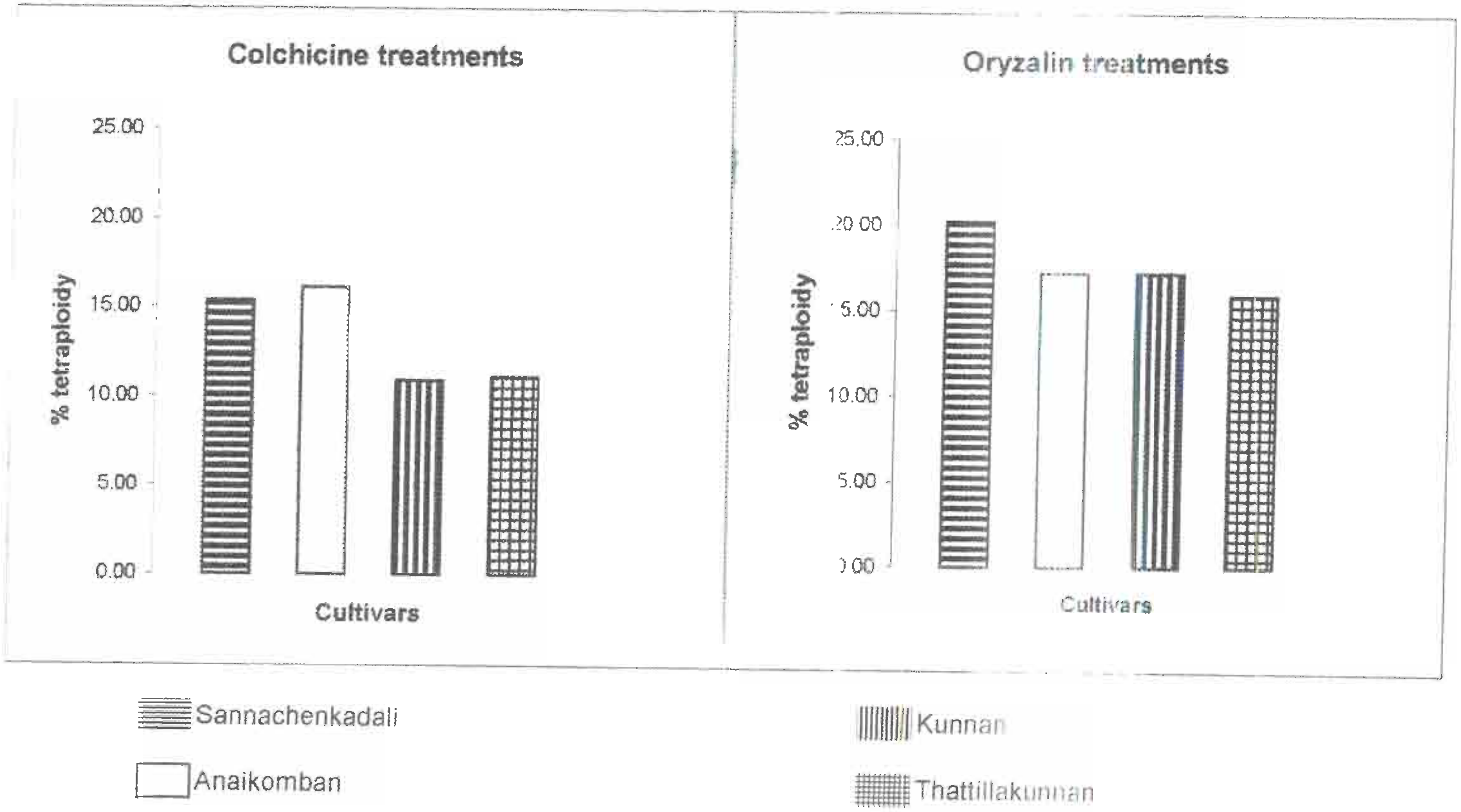
O1 - 10μM oryzalin for 3d
O2 - 20μM oryzalin for 3d
O3 - 30μM oryzalin for 3d
O4 - 40μM oryzalin for 3d
O5 - 10μM oryzalin for 6d
O6 - 20μM oryzalin for 6d

dinitroanilines to plant microtubules than that of colchicine (Hansen and Andersen (1996). Okamura (1980) observed that the affinity of oryzalin for plant tubulin was approximately ten times greater than that of colchicine.

Efficiency of oryzalin to induce tetraploidy at lower concentrations has been reported by many other earlier workers too (Strachan and Hess, 1983; Sree Ramulu *et al.*, 1991; Wan *et al.*, 1991). To be more appropriate, the higher microtubule depolymerizing effect of oryzalin is attributed to its higher affinity for plant tubulin and the stability of the oryzalin-tubulin complex. In addition, oryzalin has been reported to form a stable complex with plant tubulin, whereas colchicine does not (Hart and Sabnis, 1970; Okamura, 1980). Moreover, the interference of oryzalin with the Ca^{2+} transport systems operating in the cell organelles by way of affecting the role of Ca^{2+} in microtubule assembly (Weisenberg, 1972) might also be held responsible for the higher efficiency of oryzalin. The two-way action of oryzalin *viz.*, formation of the oryzalin-tubulin complex and the interference with Ca^{2+} transport systems might have led to a higher degree of microtubule depolymerization, resulting ultimately in higher rates of tetraploidy compared to colchicine, which has only a one-way action, *i.e.*, causing microtubule depolymerization by forming the colchicine-tubulin complex.

In the present study, the cultivars exhibited varied response with respect to the rates of tetraploidy induced (Fig. 14).

Fig. 14. Differential response of cultivars to tetraploidy induction



Differential response of banana cultivars with respect to the rates of tetraploidy induced

Source	% tetraploidy induced			
	Sannachenkadali (AA)	Anaikomban (AA)	Kunnan (AB)	Thattillakunnan (AB)
Colchicine-treated cultures	15.31	16.11	10.90	11.13
Oryzalin-treated cultures	20.19	17.19	17.20	15.89

In general, the cultivars with genomic constitution 'AA', namely, Sannachenkadali and Anaikomban have produced comparatively higher rates of tetraploidy than the cultivars with the genomic constitution 'AB', namely, Kunnan and Thattillakunnan. Such a variation in the rates of tetraploidy induced may be attributed to the variation in genomic constitution.

Occurrence of higher ploidy levels and mixoploids

In the present investigation, a few plants with stomatal densities below 8.00 stomata mm^{-2} and stomatal dimensions above $2500\mu^2$ were observed. Chromosome counting in the root tip cells of these plants revealed the existence of a very high number of chromosomes scattered throughout the cytoplasm (Plate 33). The chromosomes could not be counted exactly due to non-selective staining of the entire cytoplasm which rendered counting difficult. However, the chromosome number was approximately around 85 to 90 and hence the counts were corrected to the nearest euploid chromosome complement, *viz.*, octoploidy, as per the views of White (1928) and Cheesman (1932a, b).

In addition to the above mentioned abnormal situation, plants possessing any one or more of the following ploidy conditions were also encountered with:

a) Root tip cells of the same plant revealing the presence of the mixtures of varying chromosome numbers (22 + 44), (22 + 88) and (44 + 88) (Plate 34).

b) Leaves of the same plant possessing mixtures of stomatal densities and dimensions characteristic of diploids, tetraploids and higher ploidy levels.

c) Leaves of the same plant possessing mixtures of chloroplast densities characteristic of diploids, tetraploids and higher ploidy levels (Plates 25 and 26).

d) Leaves possessing stomatal densities and dimensions and chloroplast densities characteristic of a particular type of ploidy level while the root tip cells of the same plant possessed chromosome counts characteristic of a different ploidy level.

The octoploids and mixoploids had an overall abnormal and weak appearance and none of them survived *ex vitro* (Plate 13).

Such types of mixoploidy and chimeras are possible indicators of reversion from tetraploidy to diploidy and their occurrence has been attributed to the phenomenon of nuclear fragmentation preceding mitosis resulting from the activity of anti-mitotic agents (D'Amato *et al.*, 1980; Lupi *et al.*, 1981), chromosome losses and translocations (Karp *et al.*, 1982) or the multi-cellular origin of shoots from shoot tip and meristem tip cultures (van Duren *et al.*, 1996). Similar encounters have been reported earlier by Vakili (1967) who observed a number of colchicine treated *M. acuminata* seedlings with tetraploid appearance possessing diploid chromosome numbers in their root tip cells and vice versa. Such a phenomenon of chromosome mosaicism has also been observed in plants regenerated from anti-mitotic agent treated cultures of a wide range of plant species (Belling and Blakeslee, 1924; Muntzing, 1935; Heinz *et al.*, 1969; Bennici and D'Amato, 1978; D'Amato *et al.*, 1980).

Similar to the case with the rates of tetraploidy induced, variations also existed among the cultivars with respect to the rates of 'other' ploidy induced.

Differential response of banana cultivars with respect to the rates of 'other' ploidy induced

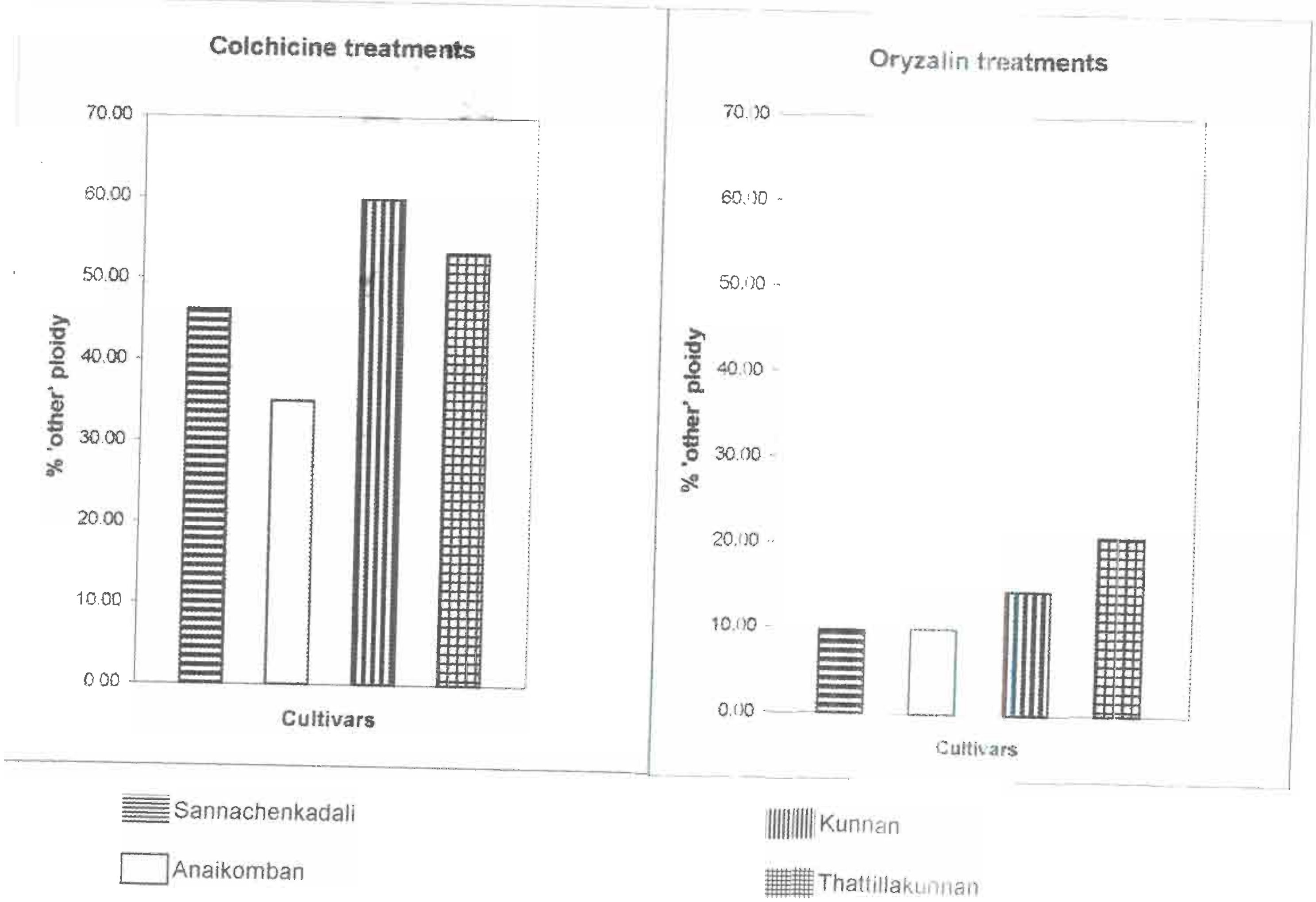
Source	% 'other' ploidy induced Sannachenkadali (AA)	Anaikomban (AA)	Kunnan (AB)	Thattillakunnan (AB)
Colchicine-treated cultures	46.12	34.94	59.88	53.30
Oryzalin-treated cultures	9.66	9.87	14.41	20.76

In general, the cultivars Sannachenkadali and Anaikomban were associated with lower rates of 'other'ploids compared with the cultivars Kunnan and Thattillakunnan (Fig. 15). The results have revealed that cultivars with genomic constitution 'AA' are more suitable for inducing tetraploidy than those with the genomic constitution 'AB'.

Phyto-toxicity of anti-mitotic agents

In the present study, certain characteristic symptoms observed in the colchicine- and oryzalin-treated cultures during their *in vitro* phase were taken as 'signs of toxicity'. Based on the severity of the symptoms, the toxic effects were categorised as highly toxic, moderately toxic, less toxic and non-toxic (Plates 2 to

Fig. 15. Differential response of cultivars to 'other' ploidy induction



4). The observations indicated that the toxic effects of colchicine were higher than those of oryzalin.

The occurrence of mixoploids and very high ploidy levels is also an indication of the phytotoxicity of the anti-mitotic agent (Hoffman and Vaughn, 1994; Van Duren *et al.*, 1996). In the present study, the overall average rate of mixoploids and 'other' category ploids induced by the colchicine treatments was very high (48.56 per cent) compared to that of the oryzalin treatments (13.68 per cent) respectively (Fig. 16). This led to the conclusion that oryzalin is less phytotoxic than colchicine.

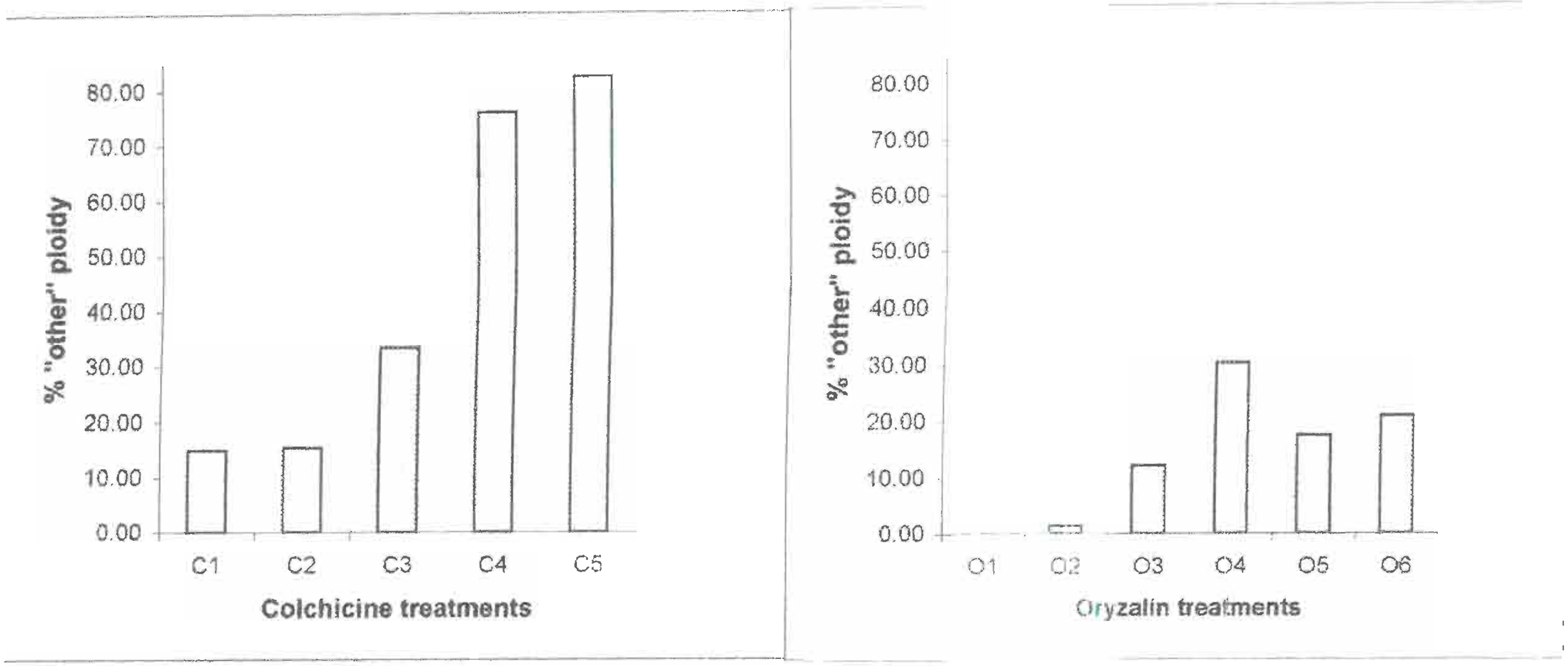
The reason underlying the higher degree of phytotoxicity associated with colchicine can be explained by the fact that although spindle toxins in general have a similar primary damage action, colchicine being a natural alkaloid, requires very different treatment conditions and concentrations from those for oryzalin (Hoffman and Vaughn, 1994). Awoleye *et al.*, (1994) also indicated the importance of applying colchicine with care and culture conditions carefully executed.

Oryzalin which was used in lower concentrations was less toxic and it produced higher numbers of solid tetraploid plants compared with colchicine. This is attributable to the fact that DNH's have besides a higher potential for *in vitro* chromosome doubling, a lower degree of toxicity, particularly at the very low concentrations needed for the chromosome doubling treatment (Hansen and Andersen, 1996).

Genetic instability

Many experimentally produced autoteraploid plants did not maintain their polyploid level and reverted to the original ploidy level. The cytological mechanisms responsible for such reversions are not yet known.

Fig. 16. Comparison of phyto-toxicity (in terms of "other" ploidy) of colchicine and oryzalin



C1 - 2.5mM colchicine for 12h
C2 - 5.0mM colchicine for 12h
C3 - 7.5mM colchicine for 12h
C4 - 2.5mM colchicine for 24h
C5 - 2.5mM colchicine for 36h
O1 - 10µM oryzalin for 3d
O2 - 20µM oryzalin for 3d
O3 - 30µM oryzalin for 3d
O4 - 40µM oryzalin for 3d
O5 - 10µM oryzalin for 6d
O6 - 20µM oryzalin for 6d

During the course of the present investigation, many of the regenerants which were categorised as tetraploids initially, when subjected to ploidy analysis after a few days, expressed features characteristic of diploids. The leaves which were initially thicker and darker green had become thinner and faded during later stages; stomatal densities and dimensions which initially reflected tetraploid status had shifted towards those characteristic of diploids. However, these plants did not express any morphological alterations which were conspicuous enough to confirm a reversion to the original diploid status. Further, the exact percentage of reversion to diploidy could not be assessed since ploidy assessment procedures proved to be very tedious and time consuming. The basis behind such vague observations can be explained by a few reports wherein similar encounters had been made.

Such reversions might be due to the more or less unstable nature of polyploid races which consequently stimulates them to revert to the normal chromosome number, both somatically and generatively (Wettstein, 1927,1928). Vakili (1967) who encountered a high degree of reversion of colchicine induced tetraploid plants of *M. acuminata* to diploidy opined that the reversion was a strong evidence for chromosomal reduction in somatic cells of those plants.

Occurrence of a higher degree of chromosome pairing other than bivalents has been quoted as one of the causes for reversion and instability (Muntzing, 1935). Gottschalk (1978) attributed the "reversion" to the action of a specific mitotic mechanism resulting in the original chromosome number or abnormally low chromosome number. Hamill *et al.* (1992) who encountered a high percentage of reversion to diploid state in colchicine-treated *in vitro* regenerated banana plants, attributed the reversion to the hetero-histont tissue resulting after colchicine treatment.

Ploidy assessment

In addition to an efficient system for induction of polyploidy, it is also vital to have an effective and reliable method for ploidy screening. In *Musa*, genome size is variable without any obvious morphological indicator of ploidy state (Ortiz and Vuylsteke, 1994). Differences between morphological traits are often greater within each ploidy level than between ploidy levels (Dessauw, 1988). Moreover, intraclonal differences are observed between mother plants and ramets. Therefore, ploidy determination cannot be based upon morphological criteria alone. Ploidy determination is traditionally done by chromosome counting (Hamill *et al.*, 1992; Osuji *et al.*, 1996). However, this is a procedure not suitable for large scale screening because of the complexities associated with the technique, namely, very low frequency of dividing cells, rare occurrence of well spread metaphase plates suitable for chromosome counting and the time needed to screen sufficient number of samples (Novak, 1992). Alternative methods of ploidy assessment involve ploidy-screening based on stomatal and chloroplast traits.

Stomata analyses

Methods to determine ploidy based on analysis of stomatal density and length have been reported (Speckmann *et al.*, 1965; Blanke *et al.*, 1994) to be easier (Sreenivasan *et al.*, 1992), but the reliablity of these methods remained doubtful until recent times, since they are greatly influenced by environmental effects . Oflate, many workers (in *Musa*.spp, as well as in other crops) have attached a considerable degree of reliability to ploidy assessment based on stomata analyses, at least as a preliminary ploidy screening technique which would help in reducing the population size that has to be subjected to further ploidy confirmation based on chromosome counting, which though remains the most reliable method, is associated with innumerable bottlenecks.

A high degree of reliability in adopting stomatal traits as a method for detecting polyploids has also been reported by many other earlier workers (Greenleaf, 1937; Hunter,1953; Dermen and Scott, 1954; Vuylsteke *et al.*, 1993; Van Duren *et al.*, 1996; Meenakumari *et al.*, 1997, 1999).

In the present investigations, plants with stomatal densities ranging between 12.00 and 19.00 stomata mm^{-2} and stomatal dimensions between 1850 and 2250 μ^2 were categorised as tetraploids. Those with stomatal densities of 39.00 – 50.00 stomata mm^{-2} and stomatal dimensions of 950-1200μ^2 were categorised as diploids, while those with densities below 8.00 stomata mm^{-2} and dimensions above 2500μ^2 were classified as octoploids. Those with mixtures of two or more of these stomatal densities and diemensions were categorised as mixoploids. The average stomatal size of the tetraploids was 1.91 times as great as that of the diploids. Subsequent chromosome counting taken up to confirm the ploidy status as assessed by the initial screening based on stomata analyses revealed that ploidy assessment based on stomata analyses was 82.19% reliable (Fig.17).

Ploidy assessment of *Musa* hybrids based on stomata analyses by Sathiamoorthy (1973) and Apshara (2000) corroborate the results of the present investigation. According to Sathiamoorthy, the mean stomatal densities of diploids and tetraploids were 40.00 and 15.20 stomata mm^{-2} respectively and according to Apshara, the values were 43.51 and 10.27 respectively. (Variations in the mean values are attributable to variations among clones). Further corroboration of the present finding is lent by Tenkouano *et al.* (1998) who observed that the average stomatal size in tetraploid accessions of *Musa* was 1.47 times as great as that in diploid accessions.

Chloroplast analyses

In the present study, it was observed that the chloroplast density per guard cell pair of the stomata was higher for all the induced tertaploids. The densities fell within a definite range for the diploids and the tetraploids (9.00-11.00 and 15.00-17.00 chloroplasts per guard cell pair respectively), enabling categorization of plants into different ploidy classes. Doubling of the ploidy level from 2x to 4x increased the chloroplast density of guard cell pair by a factor of approximately 1.70. This finding derives support from the observation of Tenkouana *et al.* (1998) who categorized *Musa* accessions with chloroplast densities of 10.00 and 15.50 chloroplasts per guard cell pair as diploids and tetraploids respectively (the average chloroplast density per guard cell pair of the tetraploids was observed to be 1.53 times as great as that in diploids).

Confirmation of ploidy status based on chromosome counting revealed that ploidy assessment based on chloroplast analyses was 95.89 % reliable (Fig. 17). The high degree of consistency of ploidy screening based on chloroplast traits has been reported by many earlier workers (Frandsen, 1968; Sree Ramulu *et al.*, 1991; Jacobs and Yoder, 1989; Compton and Veilleux, 1991; Fassuliotis and Nelson, 1992; Cardi *et al.*, 1993; McCuistion and Elmstrom, 1993; Compton *et al.*, 1996). Stonger support comes from the claims of Mattheij *et al.*(1992) that ploidy assessment through chloroplast count was as consistent as that based on flow-cytometry, based on the highly reliable results he observed with his protoplast fusion-derived interspecific hybrids of potato. Further, ploidy analysis based on chloroplast counts was claimed to be even more advantageous than that based on flow-cytometry due to demerits such as sophistication and high expenses associated with flow-cytometry.

Chromosome counting

Though chromosome counting undoubtedly is the most reliable ploidy assessment method, in *Musa* spp. it is associated with innumerable difficulties. The bottlenecks encountered in this respect during the course of the present investigation are discussed below:

1) Non availability of sufficient number of root tips for analysis: This is attributable to the sparse root producing characteristic of tetraploids (Vakili, 1967).
2) Non-selective staining of tissues: This resulted in staining of the entire cytoplasm instead of selective staining of chromosomes alone (Plates 29, 31 and 33). This rendered identification and counting of the chromosomes very difficult (Osuji *et al.*,1996).
3) Among a large number of specimens, very few showed the presence of mitosis. This can be explained by the fact that *Musa* spp. are associated with very low frequencies of dividing cells as expressed by Osuji *et al.*, (1996) who estimated the mitotic index in root tips to be as low as 1 % or less.
4) Difficulty in identification of chromosomes: The chromosomes appeared as minute dots even at magnifications of X1000 and X1500 (Plates 33).The reason for this is the very small size of the *Musa* chromosome (1-2μm), when observed at mitotic metaphase (Dolezel *et al.*, 1994). The average nuclear genome size in *Musa* is only 50Mbp DNA per chromosome. Further, Osuji *et al.*, (1996) attributed the difficulty in identifying chromosomes to the rare occurrence of well spread metaphase plates suitable for chromosome counting.
5) Poor staining of cells: Most of the cells in a prepared specimen remained unstained, appearing as empty cells (Plate 34). This is

attributable to the rigid cell walls of *Musa* spp, which prevent staining. Further, presence of remnants of cell wall and cytoplasm makes high resolution chromosome analysis non reliable. (Dolezel *et al.*, 1994).

6) Presence of a number of cellular structures which were either unknown or unidentifiable (Plates 45 and 46). Similar has been the case with many of the earlier reports on *Musa* karyology (Dolezel *et al.*, 1994,1997; Jenny *et al.*, 1997; Horry *et al.*, 1998).

Due to such problems, the chromosome numbers in cases where exact counting could not be done were corrected to the nearest euploid number, following the suggestions derived from earlier identical works (White, 1928; Cheesman, 1932). Further, during such cases of uncertainty, chloroplast counts were taken as a supporting evidence, since they have been associated with a very high degree of reliability.

In the present investigations, out of the plants regenerated from colchicine treated cultures of the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan, 15.31%, 16.11%, 10.90% and 11.13% respectively possessed the tetraploid chromosome complement (2n = 4x = 44). Out of the plants regenerated from oryzalin-treated cultures, 20.19%, 17.19%, 17.20% and 15.89% of the four cultivars respectively possessed the tetraploid chromosome complement.

CYTOLOGICAL CONSEQUENCES OF INDUCED POLYPLOIDY

Increased cell size

Control and treated cells were compared microscopically for attributes such as size, cellular organisation etc. Significant morphological changes were recorded in the treated cells. One of the most conspicuous response was a marked change in the overall size (Plate 43). Increase in cell size has been reported as a consequence of polyploidization (Muntzing, 1935). However, not all the cells responded to colchicine or oryzalin, and those that were affected varied in their degree of responses. This explains why all the cells in a specimen were not of a similar size and morphology, as observed in the present work.

Increased nuclear material

In the present investigation, the INV's of the induced tetraploids were higher (ranging between $1098.32\mu^3$ and $1420.32\mu^3$) than those of the control diploids (ranging between $412.69\mu^3$ and $422.14\mu^3$) (Plate 40). It is a well-established fact that the size of the cell and the nucleus is positively correlated with ploidy (Dodds and Simmonds, 1948a, b).

A doubling of the chromosome number is believed to have led to an increase in the DNA content which has been reflected by the increased INV. Further, reports of Berger (1941), Dodds and Simmods (1946), Dessauw (1988) and Tenkouana *et al.*, 1998 support the view that increase in cell and nuclear size is characteristic of ployploid cells and could be used as an indication of polyplodization.

Similar was the view of Walne (1966) which revealed a substantial increase in the amount of DNA accompanying a corresponding increase in cell and nuclear dimensions in *Chlamydomonas*. More convincing reports (Avanzi *et al.*, 1966; Yamaguchi and Tsunoda , 1969; Katayama , 1971; Sparrow *et al.*, 1975; Das and Mallick, 1989 b) reveal the fact that the INV is directly related to the DNA content of the nucleus.

Orientation of chromosomes

Microscopic examination of the cells of anti-mitotic agent treated cultures revealed the presence of chromosomes more or less scattered in many of the cells (Plates 27 to 30; Plate 33). This might be the result of the impairment of the spindle fibres which are responsible for holding the chromosomes in the equatorial plate, *ie.*, the metaphase plate in normal mitosis. Cleary and Hardham (1987) also observed that oryzalin treated cells had chromosomes randomly distributed throughout.

Multinucleate condition

Some of the cells (especially those subjected to high concentration and prolonged colchicine treatments, *viz.*, 7.5mM for 12h, 2.5mM for 24h, 2.5mM for 36h) manifested an apparent multi-nucleate condition (Plate 44). Constriction of an enlarged, convoluted nucleus at several sites could have created the illusion of a multi-nucleate condition, the interconnections often visible only with proper staining. Further, constriction and subsequent abscission at some sites might have accounted for the origin of nuclear bodies of variable sizes. Finally, recurring mitotic arrests and the formation of restitution nuclei involving circumvention of anaphase and telophase leading to highly polyploid cells explain both real and apparent multi nucleate conditions (Walne, 1966).

Excessive accumulation and extrusion of cellular components

Microscopic observation of cultures subjected to prolonged colchicine treatments (24h and 36h) revealed the presence of cells with seemingly extruded particles (Plate 45). This might be due to the increased nuclear material resulting from accumulated metaphases resulting from prolonged colchicine activity exerting a pressure on the cell wall leading to cell wall breakage followed by extrusion of cellular ingredients. A similar kind of observation has been reported by Walne (1966) in *Chlamydomonas*, wherein prolonged exposure (48h, 72h) to colchicine led to surface damage resulting in extruded particles. This was associated with the breakdown of some membranous components of the cells.

Cytological observations in the cultures treated with higher concentrations of colchicine and those involving prolonged treatments revealed also the presence of large amounts of cellular components (Plate 46). This is quite possibly an indication of reduced metabolic activity in the treated cells resulting from an altered or interrupted mitosis. The reduction in normal metabolic activity would have prevented the breakdown of lipid substances, resulting in excessive accumulation of the same (Walne, 1966).

PHYSIOLOGICAL CONSEQUENCES OF INDUCED POLYPLOIDY

Retarded and accelerated growth rates

The induced tetraploids derived from the colchicine- and oryzalin-treated cultures were associated with an initial phase of retarded growth (Plate 42) followed by an increase in the vigour after a certain stage. The initial retarded growth phase (up to two months after transfer to *ex vitro* environment) was reflected in terms of lower *ex vitro* survival rates, shorter and thinner pseudostems, larger phyllochron values (due to longer time intervals between leaf emergence), smaller leaves and lower crop growth rates. The vigorous growth during the later phase (from around the third month after transfer to *ex vitro* environment) was reflected in terms of taller and thicker pseudostems, lower phyllochron values (due to shorter time intervals between leaf emergence), larger leaves and higher crop growth rate values (Plates 35 to 38).

Vakili (1967) attributed poor survival rates coupled with poor initial growth characteristics followed by vigorous growth at later stages among his colchicine-induced tetraploids derived from diploid clones of *M. acuminata and M. balbisiana,* to the poor rooting characteristics of these plants. The slower rate of development may be attributed to a decreased rate of cell division, since polyploidy has been reported to have a marked effect on the rapidity of cell division (Wettstein, 1924a). Further, these ploidy-altered plants might have reacted in a new way both with the normal environment and to changes of the normal environmental conditions leading to lower survival rates (Muntzing, 1935; Song *et al.*, 1997). Similar observations have also been reported by many other earlier workers (Gates, 1913, 1915; Nilsson, 1920; Stomps,1925; Honing,1928; Navashin, 1929; Lesley and Lesley,1930; Kostoff and Kendall,1934).

In the present investigations also, the anti-mitotic agents might have lowered the rates of cell division which might have led to an extension in the time interval between leaf emergence and reduction in the growth rates during the initial phase. This is reflected by the phyllochron values of the induced tetraploids which were higher than those of the corresponding diploids, at transfer to *ex vitro* environment and after two months, followed by lower phyllochron values at the fourth month after transfer. Similarly, the crop growth rates of the induced tetraploids were lower than those of the corrresponding diploids, at transfer to *ex vitro* environment and after two months, and were higher at the fourth month after transfer. A number of physiological consequences of chromosome doubling other than changed rates of cell division have been reported, which also might have attributed to the retarded growth rates (Wettstein, 1924b).

The photosynthetic efficiencies of the induced tetraploids were higher than those of the corresponding diploids, four months after transfer to *ex vitro* environment. This can be regarded as a polyploidy-associated physiological alteration, as expressed by D'Amato (1989). The increased chlorophyll contents coupled with increased RNA synthesis are possible reasons for the increased photosynthetic rates (Loveys and Bird, 1973) which in turn might have led to the increased growth rates. The larger leaves of the induced tetraploids might have also led to the increased photosynthetic efficiencies. Further, the ploidy-altered plants which are believed to react in a new way both with the normal environment and to changes of the normal environmental conditions are assumed to have attained higher growth rates once they had acclimatized to the environment.

Reduced mechanical strength of leaves

In spite of their robust plant parts, induced polyploids have been associated with poor agronomic performance (Stover and Buddenhagen, 1986; Singh *et al.*, 1990; van Duren *et al.*, 1996). The poor mechanical strength of the leaves of polyploids is one such trait contributing to the poor overall agronomic performance. Leaf lamina weights and percentage dry weights of petioles have been reported to be traits of immense value in banana, since these are the ones which regulate the mechanical strength of the leaves (Simmonds, 1948). The tendency of the petiole to bend and break depends not only on the strength of the petiole but also on the strain imposed upon it. Lamina weight is one of the physical strains imposed on the petiole.

In the present study, the leaf lamina weights of all the induced tetraploids were higher than those of the untreated control diploids. On the other hand, the percentage dry weights of petioles of the induced tetraploids were lower than those of the untreated control. The physiological basis underlying this kind of a trend has been explained by Simmonds (1948). The leaf of *Musa* is one of the largest to be found in any plant and may attain about $2m^2$ in area and over a kilogram weight. The strains imposed on the petioles must be great, but nevertheless, the leaf of the ordinary wild diploid banana is mechanically well adjusted to its environment, and even in high winds, breakage at the petiole is rarely or never seen. Instead, the lamina tears and the leaf remains in position even if tattered (Skutch, 1927). In polyploids, however, this mechanical adjustment is lost. The leaves of polyploids droop more than in diploids and are more liable to breakage of the petiole near the base of the lamina.

Thus, flaccidity of the leaf is roughly proportional to ploidy (upto the pentaploid level, at least). It is positively correlated with the leaf lamina weight and negatively correlated with dry matter content (Simmonds, 1948b). The leaves of polyploids have been reported to be more flaccid than those of diploids, and this is correlated with and believed to result from a corresponding decrease in percentage dry weight of the petiole (Simmonds, 1948a). The percentage dry weight of the petiole might be taken as a measure of its content of material having, at least potentially, a mechanical function. This mechanical content has been reported to decline with increasing ploidy.

THE MORPHOLOGICAL CONSEQUENCES OF INDUCED POLYPLOIDY

Morphological gigantism

The plant parts of the induced tetraploids derived in the present investigations, which were initially smaller, attained morphological gigantism (expressed as taller and thicker pseudostems and larger leaves) after a certain period of growth (Plates 35 to 38). The basis behind such an observation can be explained by the opinion of Muntzing (1935) and D'Amato (1989) who reported that the most striking properties of experimental polyploids were no doubt their morphological gigas characters.

Such morphological alterations have been believed to result mainly from the increased cell size. Meenakumari *et al.* (1999) also supports the view that induced tetraploidy was associated with a significant increase in plant height, leaf area and leaf thickness.

Characteristic morphological alterations associated with ploidy

Thicker and darker green leaves

Leaves of the induced tetraploids derived in the present study were comparatively thicker (Plate 47) and darker green than those of their diploid counterparts. The enhanced leaf thickness is believed to have resulted from increased cell size and a corresponding increase in palisade and spongy cells. The darker green colour is attributable to the increased chlorophyll contents of the induced tetraploids (ranging between 2.79mgg^{-1} and 3.21mgg^{-1}) compared to the control diploids (ranging between 2.11 and 2.24 mgg^{-1}). This increased chlorophyll content in turn is attributable to the doubling or an increase in the chlorophyll content itself (Vakili, 1967)

Lower LWR

All the tetraploids recorded lower LWR than the untreated control. The average LWR of the tetraploids was approximately 1.6 times lower than that of the control. These lower LWR values are due to the lesser difference between the length and the breadth of the leaves of the tetraploids unlike the higher difference between leaf length and breadth observed in the diploids. There existed differences between the cultivars the LWR values of the tetraploids induced from the cultivars Sannachenkadali and Anaikomban being slightly lower than those induced from the cultivars Kunnan and Tattilakunnan. This reveals the differential response of different genomic constitutions to the impact of ploidy upon the leaf LWR.

The present finding is in agreement with that of Simmonds (1952) according to whom leaf LWR apparently fell as ploidy rose in *M. acuminata* series. Simmonds (1948) observed that the effect of ploidy on leaf index was characteristic of a number of factors, one of them being the differential restriction of development of the two lamina halves of the banana leaf. A similar type of observation was made by Compton *et al.* (1996) in watermelon, wherein, the leaf size and LWR proved to be good indicators of ploidy.

Ununiform lamina development

Majority of the control diploids had the right lamina-half broader than the left. On the other hand, in a number of tetraploids the right lamina was slightly narrower and was associated with some distortion near the margin (Plate 48). Skutch (1930) opined that such type of variations in the leaf morphology were connected with increased thickness of the lamina and a certain degree of lack of co-ordination between the growth of the lamina and the development of the marginal vein.

Such types of characteristic morphological changes associated with ploidy have also been reported in grapes, wherein, among shoots arising from colchicine-treated buds, fully tetraploid shoots were diagnosed based on the characteristic U-shaped base at the point of blade and petiole attachment. This change in the shape of the curvature at the blade and petiole attachment has been reported to serve as a highly reliable morphological indicator of fully tetraploid shoots in grapes (Dermen , 1954).

THE BIOCHEMICAL CONSEQUENCES OF INDUCED POLYPLOIDY

Higher chlorophyll content

In the present investigations, the induced tetraploids were found to possess higher chlorophyll contents (ranging between 2.79mgg^{-1} and 3.21mgg^{-1}) compared to their corresponding diploids, (ranging between 2.11 and 2.24 mgg^{-1}). This was visually expressed by the darker green leaves of the tetraploids compared to those of the corresponding diploids (Plate 35). Similar were the observations made by Dermen (1954) in grapes. Increase in the number of chloroplasts (as evidenced by the chloroplast counts in the guard cell pairs of stomata as a measure of ploidy) might have attributed to the increased chlorophyll contents.

Higher anthocyanin content

The general patterns of anthocyanin pigmentation in banana clones with red-pigmented foliage and pseudostems are characteristic of a species/clone and are therefore of considerable taxonomic value. In the present study, the anthocyanin contents of the tetraploids induced from the cv. Sannachenkadali (which is characterized by red pigmented foliage and pseudostem) were higher (the ranges of the lamina and petiolar anthocyanin contents being 229.71 to 249.24mg100g-1 and 254.18 to 271.49mg100g-1 respectively) than those of the corresponding diploids (the lamina and petiolar anthocyanin contents being 136.43 and 173.14mg100g-1 respectively). These higher anthocyanin contents have been visually expressed by the highly pigmented areas on the leaves of the induced tetraploids (Plate 39).

This finding is in agreement with that of Vakili (1967) who revealed the relationship between ploidy and leaf pigmentation based on his experimental colchiploids of certain clones of *M.acuminata* subsp.*microcarpa* which had red pigmented foliage. In the tetraploids, the colour of the foliage increased in intensity. The greater colour intensity might be assumed to caused by a higher concentration of anthocyanin pigment in the vacuole of each cell rather than a doubling of the number of cells with coloured vacuoles.

SIGNIFICANCE OF THE INDUCED TETRAPLOIDS

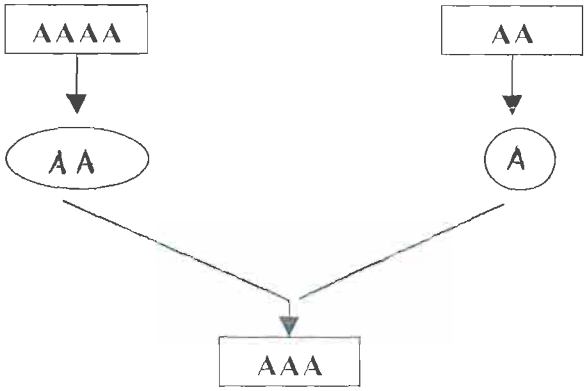
The tetraploids derived in the present investigations gain importance since they must possibly be carriers of the desirable genes respective of the original diploids from which they have been derived (fusarial wilt resistance of Sannachenkadali and Anaikomban; nematode resistance of Anaikomban, Kunnan and Thattillakunnan). This makes them invaluable for the production of secondary triploids.

The tetraploids are now in their vegetative phase. Further evaluation of the induced tetraploids to assess their genomic constitution, fertility status, disease resistance, economic traits, crossability with diploids, etc. would give a better picture of the rate of success of the techniques adopted in the present investigation as well as the actual significance of the induced tetraploids themselves.

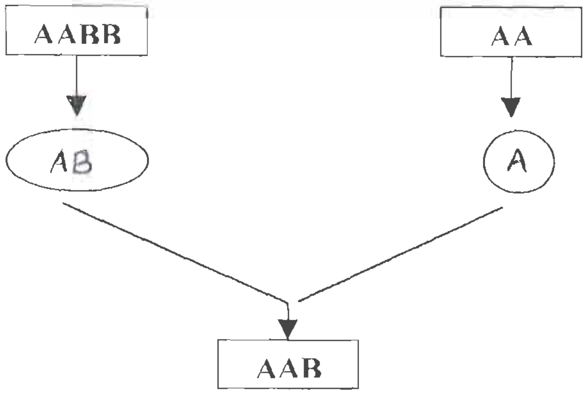
Once the potential tetraploids are identified and their fertility status assessed, those which are female fertile may be utilized as female parents in future breeding programmes. They may be crossed with the potential synthetic diploids which have been developed at the Tamil Nadu Agricultural University (Sathiamoorthy, 1987) to produce synthetic triiploids as indicated below.

Fig. 18. Possibilities of utilization of the induced tetraploids in future breeding programmes aimed at synthesizing secondary triploids

(a) Induced tetraploid x Potential synthetic diploid



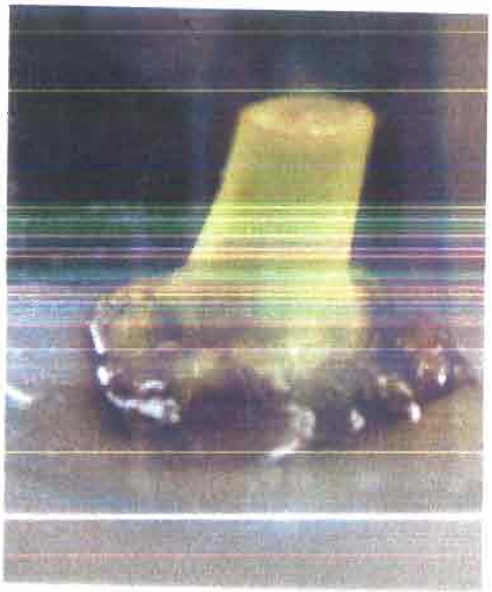
(b) Induced tetraploid x Potential synthetic diploid



FUTURE PROSPECTS

As obvious from previous reports and the present investigations, the upliftment of *Musa* karyology needs to be given utmost importance. In spite of invention of improved techniques such as the 'flow-cytometry' (FCM) for easier, rapid and accurate ploidy assessment, difficulties still exist in ploidy- and karyology-related research work, since such facilities have not been made easily available. One of the main reasons for this is the high initial expenses involved. The innumerable efforts taken during the course of the present investigation to access the technique of flow-cytometry proved futile, since this technique has not yet been applied to plant research in India. At present, very few institutions in India (The Indian Institute of Sciences, Bangalore and The Institute of Nuclear Medicine and Allied sciences, New Delhi being two of them) have the FCM facility being applied only to medical research. Making this facility easily available for plant research would do a great deal to improve the state of *Musa* karyology which is very essential for *Musa* improvement.

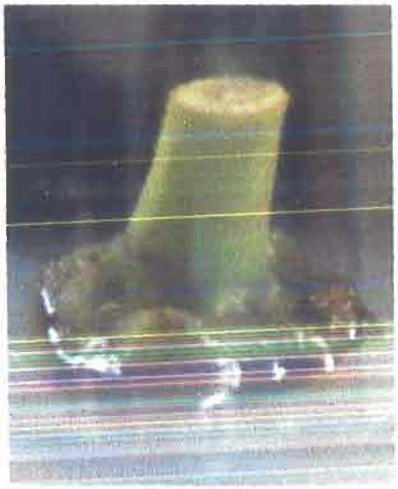
Plate 1. Shoot tip greening



At inoculation

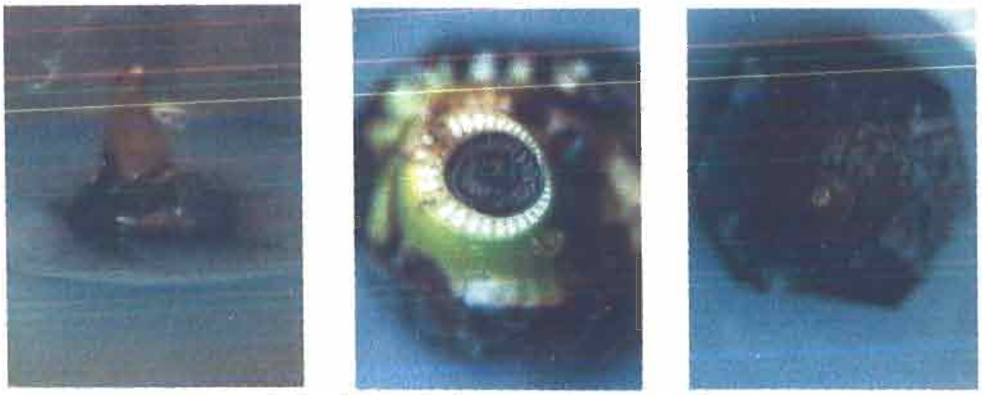
Greening

Plate 2. Phyto-toxicity in primary culture phase



Control

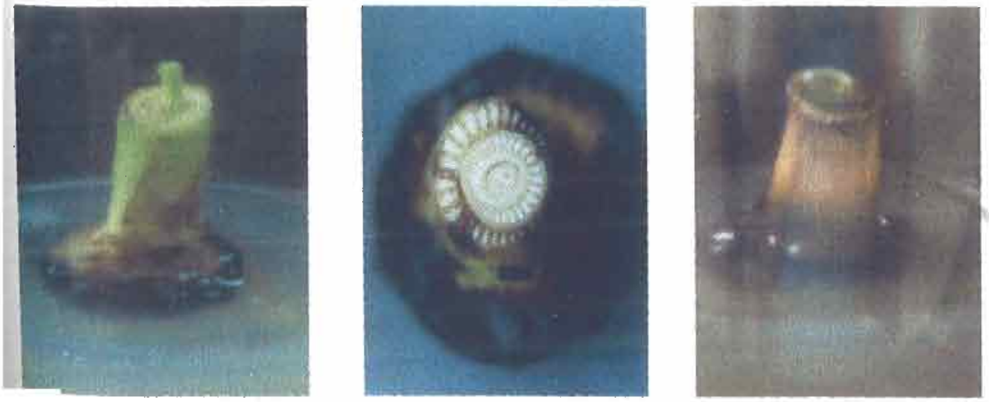
Colchicine treatments - Highly toxic



C4 (2.5mM for 24h)

C5 (2.5mM for 36h)

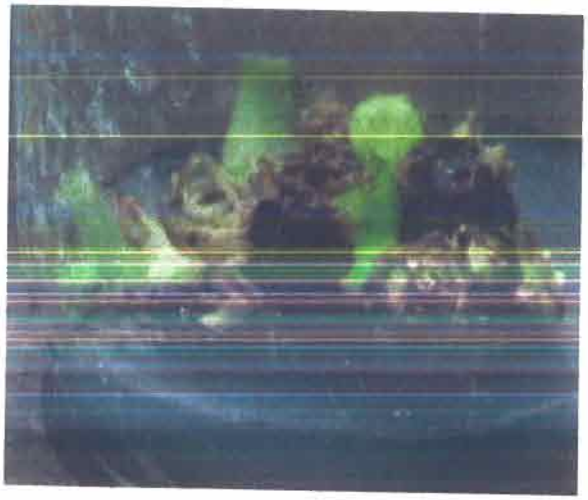
Oryzalin treatments - Less toxic



O5 (10µM for 6d)

O6 (20µM for 6d)

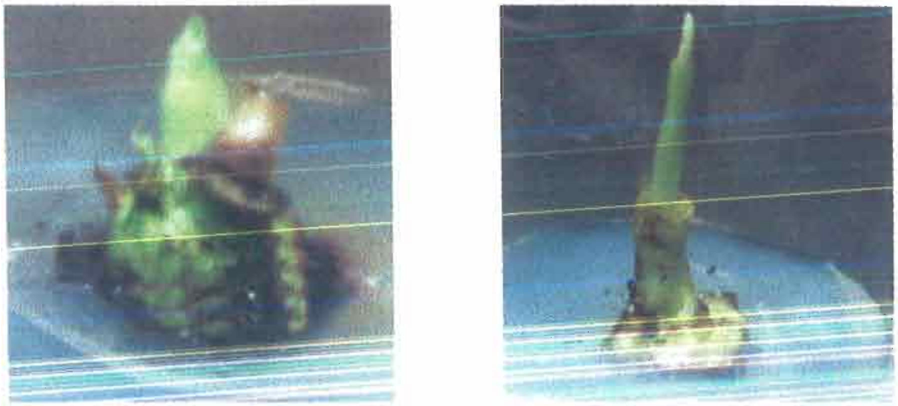
Plate 3. Phyto-toxicity in multiple bud differentiation stage



Highly toxic

Less toxic

Plate 4 . Phyto-toxicity at shoot proliferation stage



Control

Colchicine-treated cultures

C3 (7.5mM for 12h)
Moderately toxic

C4 (2.5mM for 24h)
Highly toxic

C5 (2.5mM for 36h)
Highly toxic

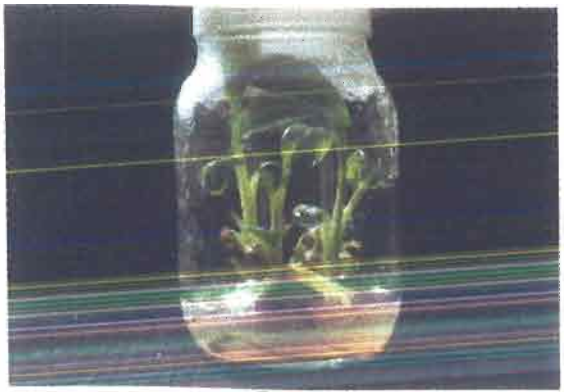
Oryzalin-treated cultures

O4 (40μM for 3d)
Less toxic

O5 (10μM for 6d)
Moderately toxic

O6 (20μM for 6d)
Moderately toxic

Plate 5 . Multiple shoot regeneration in colchicine-treated cultures



Control

C1(2.5mM for 12h)

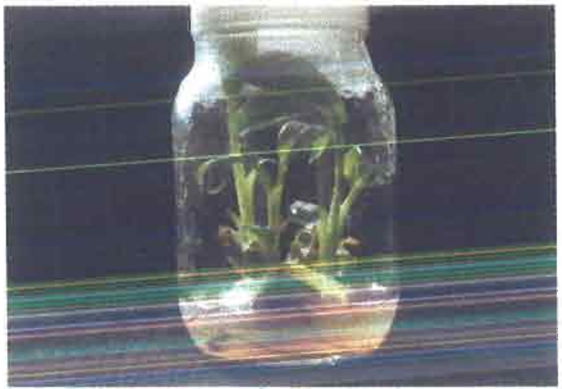
C2(5.0mM for 12h)

C3(7.5mM for 12h)

C4(2.5mM for 12h)

C5(2.5mM for 36h)

Plate 6 . Multiple shoot regeneration in oryzalin-treated cultures



Control

O1 (10µM for 3d)

O2 (20µM for 3d)

O3 (30µM for 3d)

O4 (40µM for 3d)

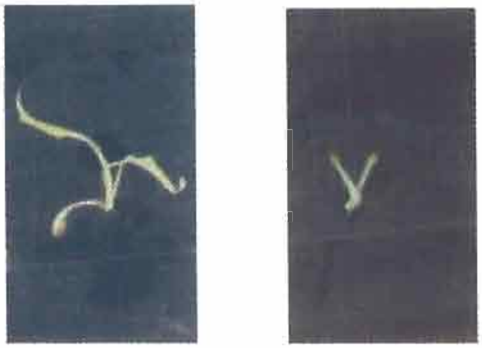
O5 (10µM for 6d)

O6 (20µM for 6d)

Plate 7 . Microshoot elongation in colchicine-treated cultures

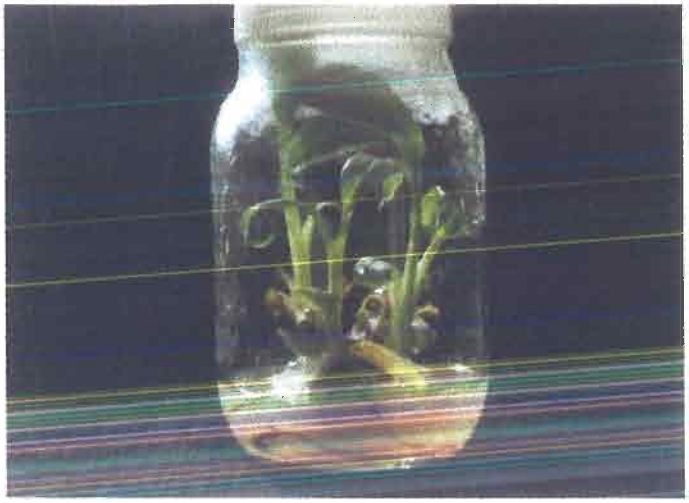
Control

C1 (2.5mM for 12h) C2 (5.0mM for 12h) C3 (7.5mM for 12h)



C4 (2.5mM for 24h) C5 (2.5mM for 36h)

Plate 8 . Microshoot elongation in oryzalin-treated cultures



Control

O1 (10µM for 3d)

O2 (20µM for 3d)

O3 (30µM for 3d)

O4 (40µM for 3d)

O5 (10µM for 6d)

O6 (20µM for 6d)

Plate 9. Number of roots in colchicine-treated cultures


Control


C1 (2.5mM for 12h)


C2 (5.0mM for 12h)


C3 (7.5mM for 12h)


C4 (2.5mM for 24h)


C5 (2.5mM for 36h)

Plate 10. Number of roots in oryzalin-treated cultures

Control

O1 (10µM for 3d)

O2 (20µM for 3d)

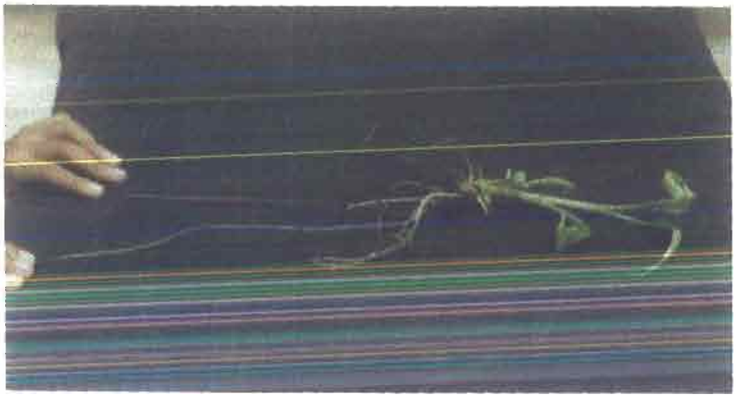
O3 (30µM for 3d)

O4 (40µM for 3d)

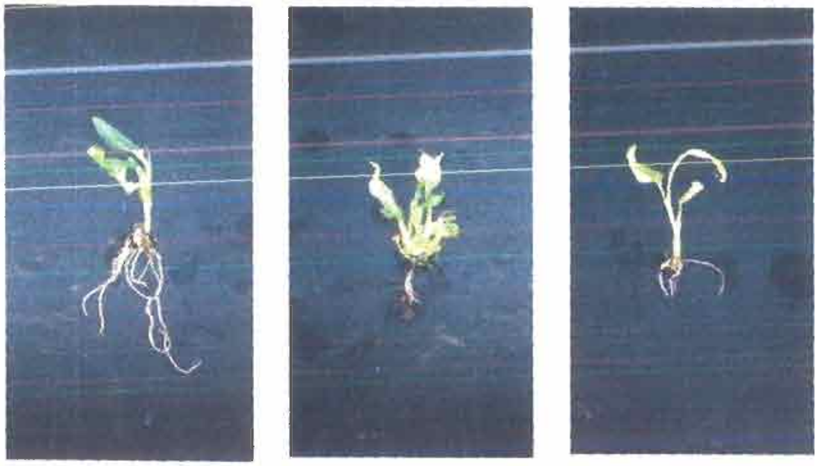
O5 (10µM for 6d)

O6 (20µM for 6d)

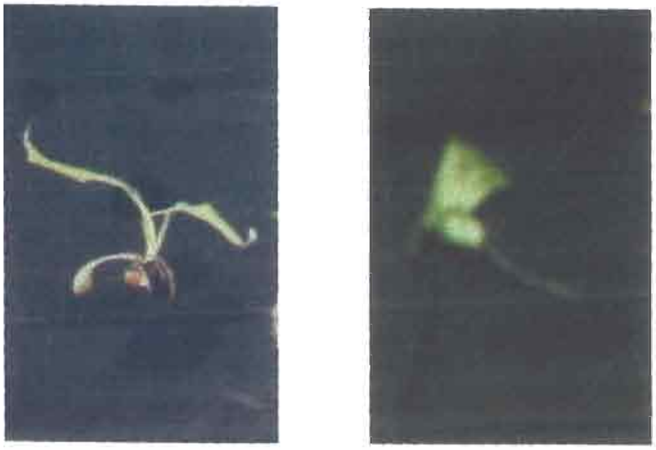
Plate 11. Root length in colchicine-treated cultures



Control

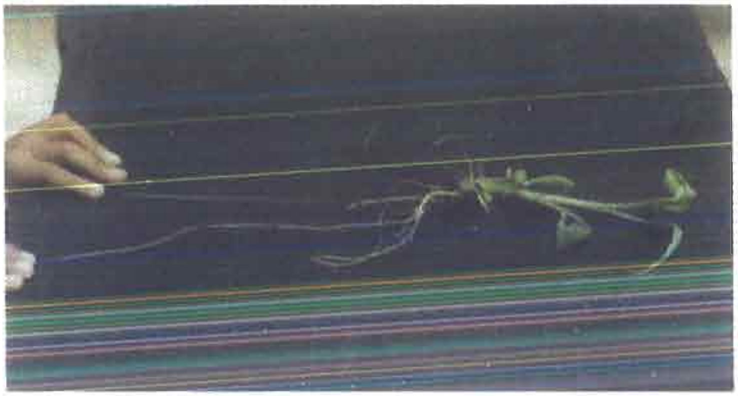


C1 (2.5mM for 12h) C2 (5.0mM for 12h) C3 (7.5mM for 12h)

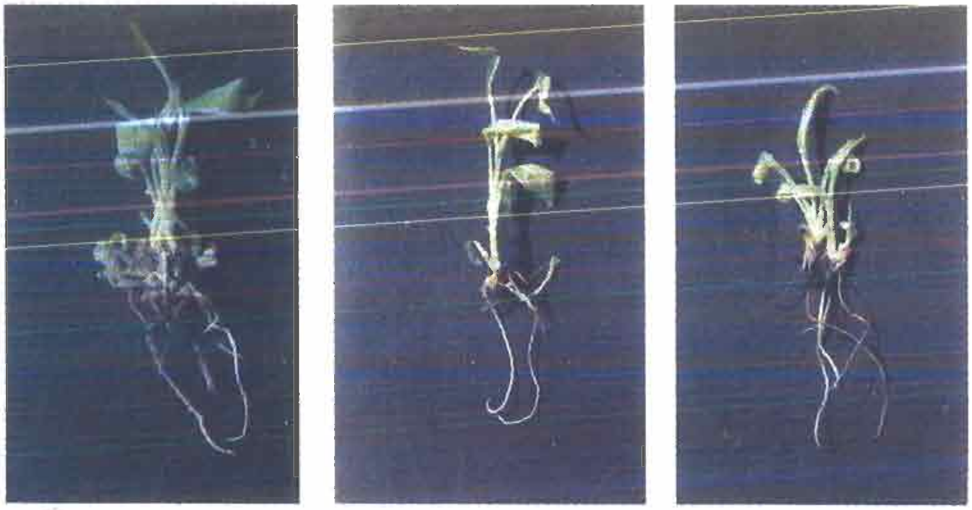


C4 (2.5mM for 24h) C5 (2.5mM for 36h)

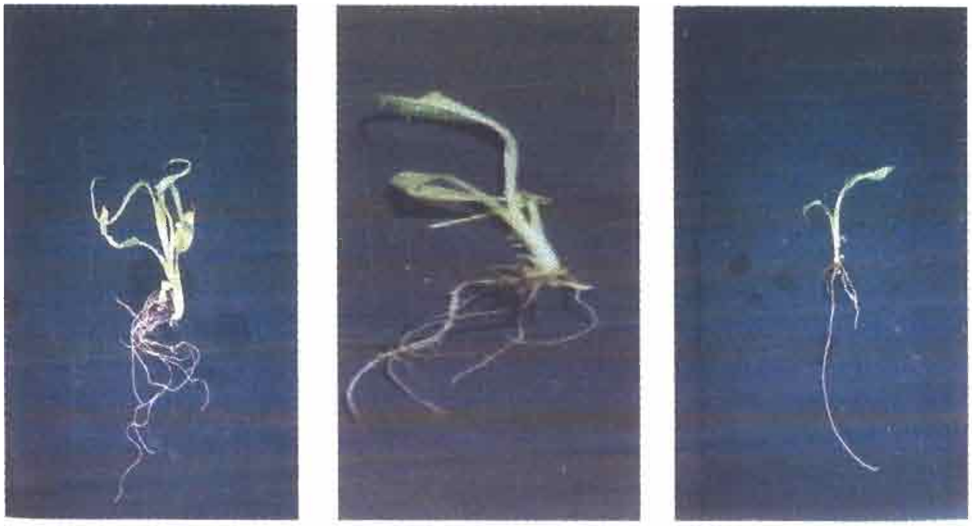
Plate 12. Root length in oryzalin-treated cultures



Control



O1 (10µM for 3d) O2 (20µM for 3d) O3 (30µM for 3d)



O4 (40µM for 3d) O5 (10µM for 6d) O6 (20µM for 6d)

Plate 13. Poor *ex vitro* survival of 'other' ploids

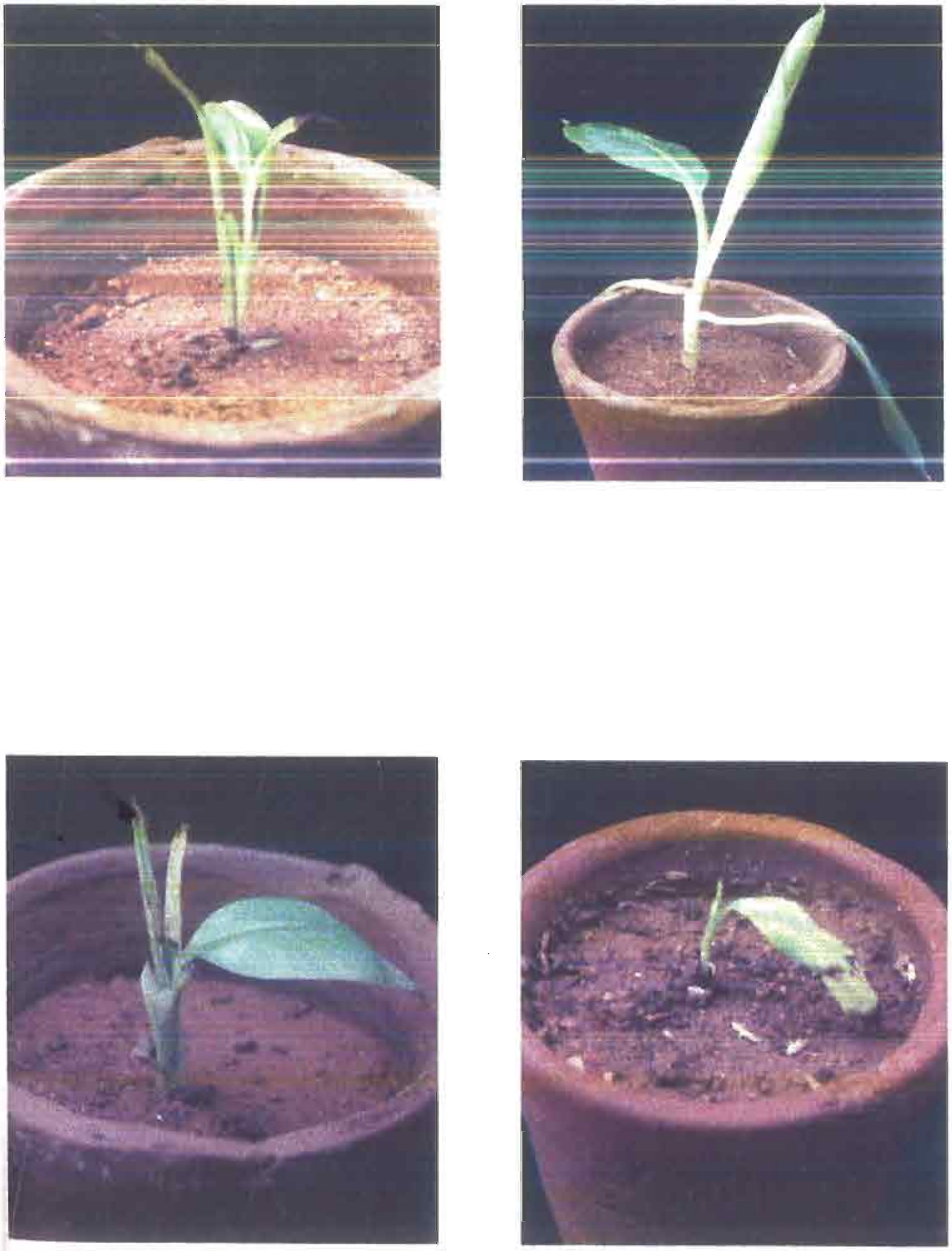
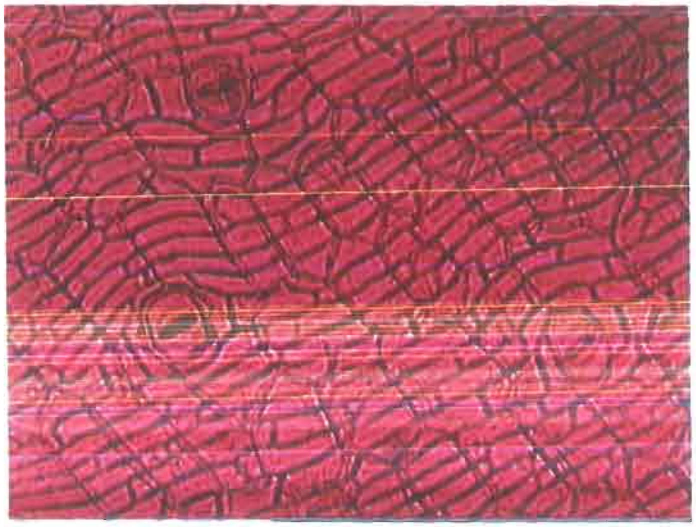
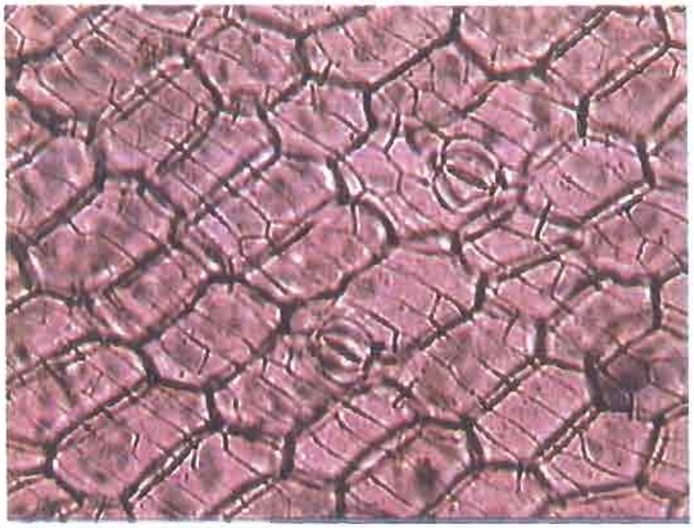


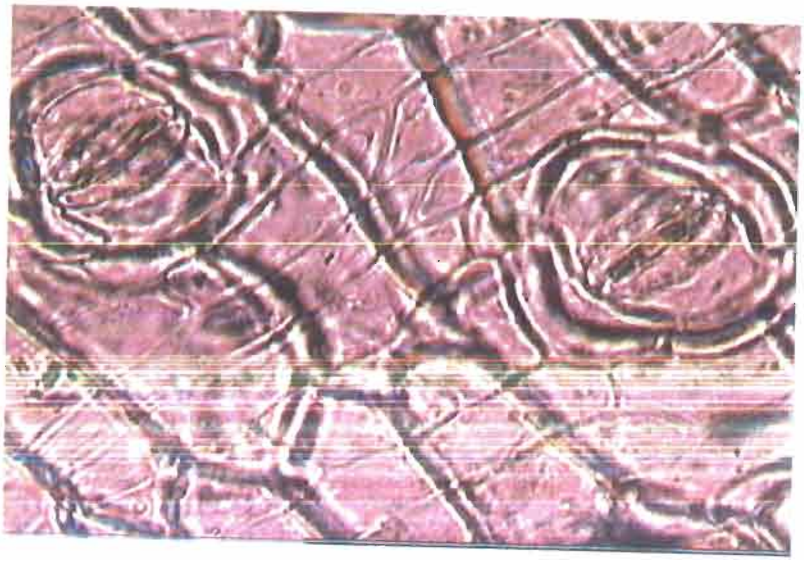
Plate 14. Stomatal density in induced tetraploid regenerants



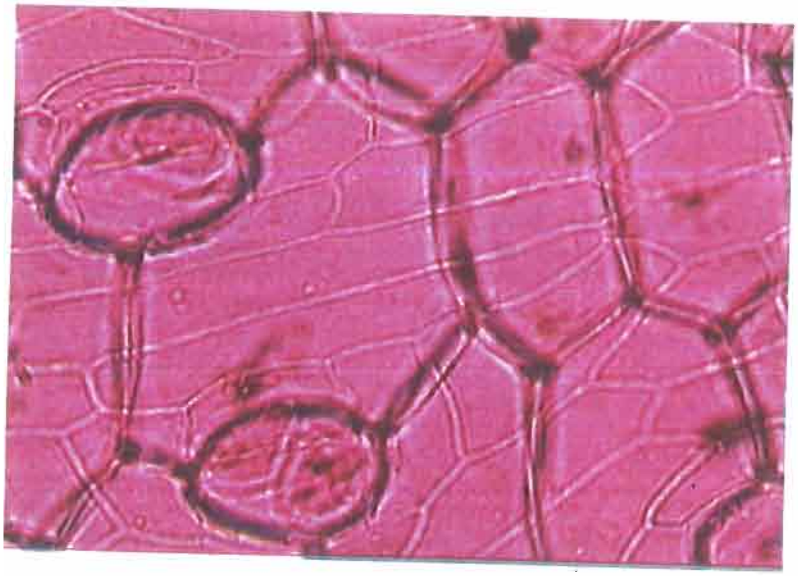
cv. Sannachenkadali



cv. Anaikomban

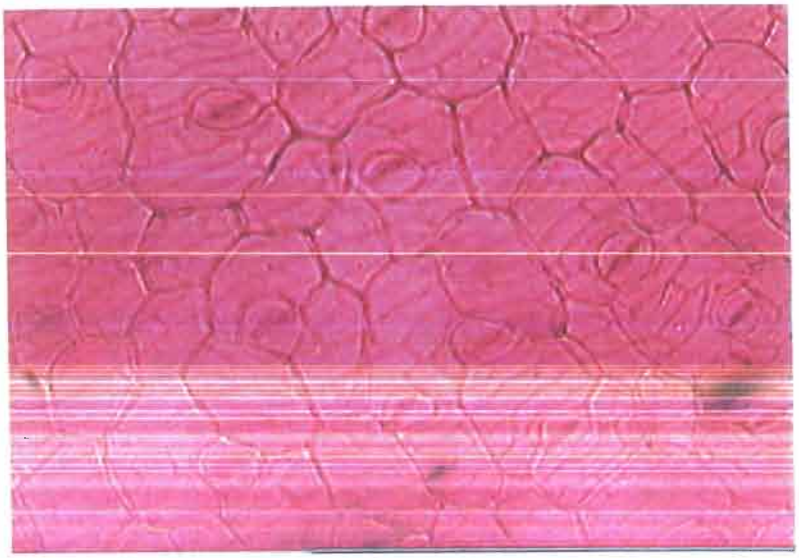


cv. Kunnan

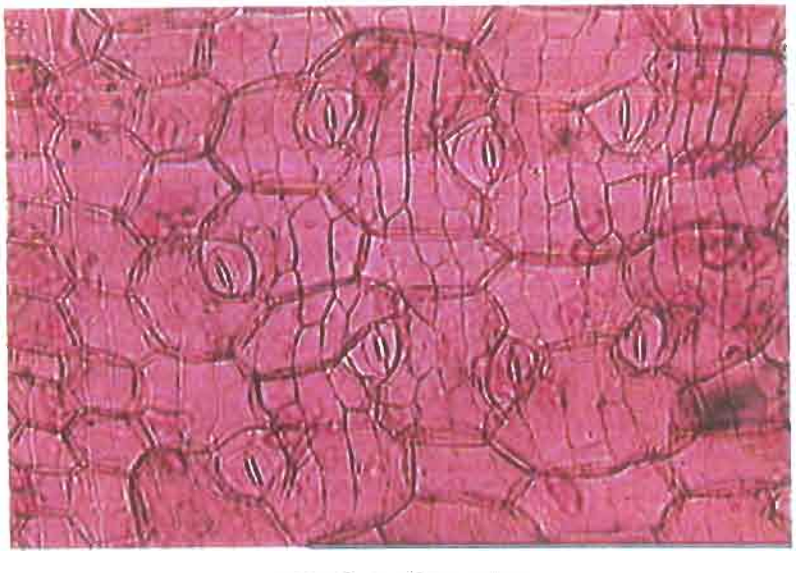


cv. Thattillakunnan

Plate 15. Stomatal density in induced tetraploid regenerants

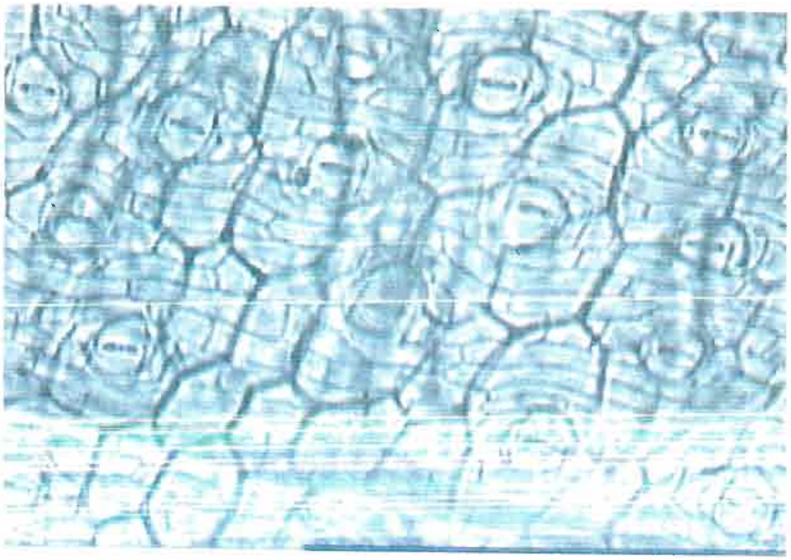


cv. Sannachenkadali

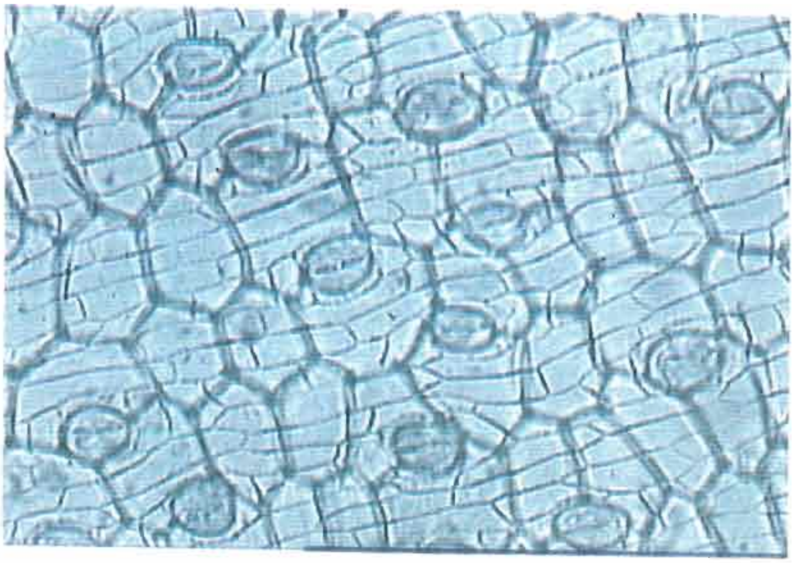


cv. Anaikomban

Plate 16. Stomatal density in diploid regenerants



cv. Kunnan



cv. Thattillakunnan

Plate 17. Stomatal density in diploid regenerants

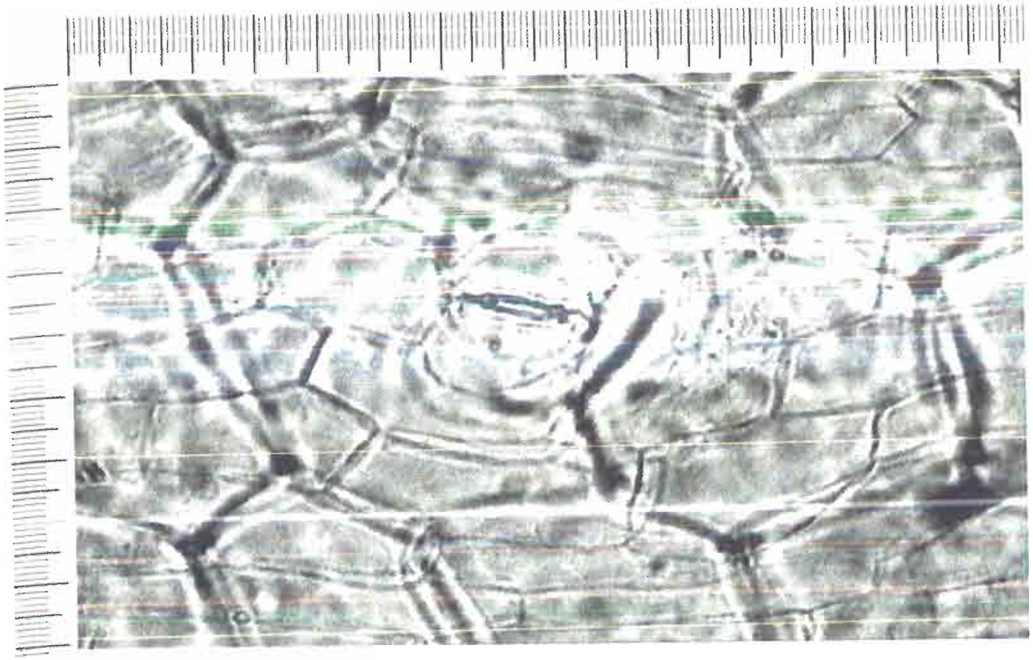
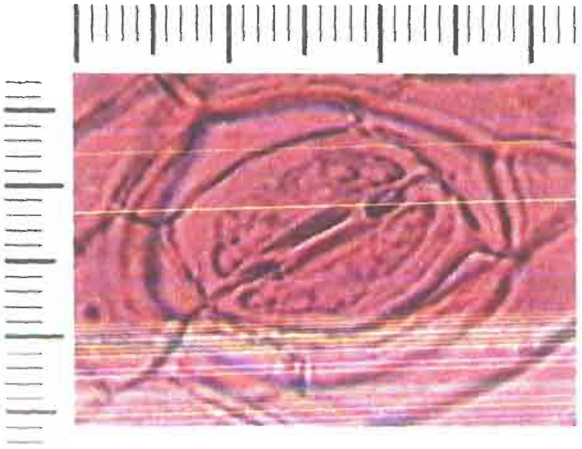
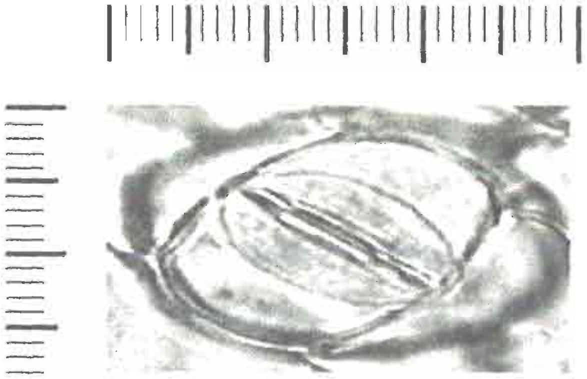


Plate 18. Stomatal density in an octoploid regenerant
(cv. Kunnan)

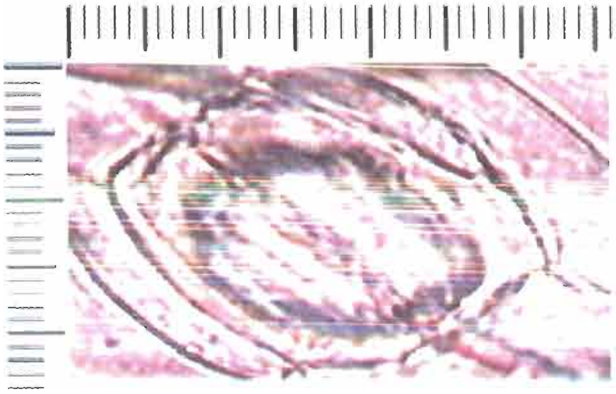


cv. Sannachenkadali



cv. Anaikomban

Plate 19. Size of stomata in induced tetraploid regenerants (1division = 2.45μ)



cv. Kunnan

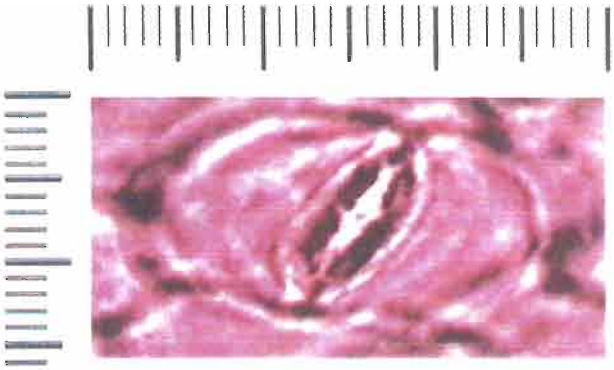
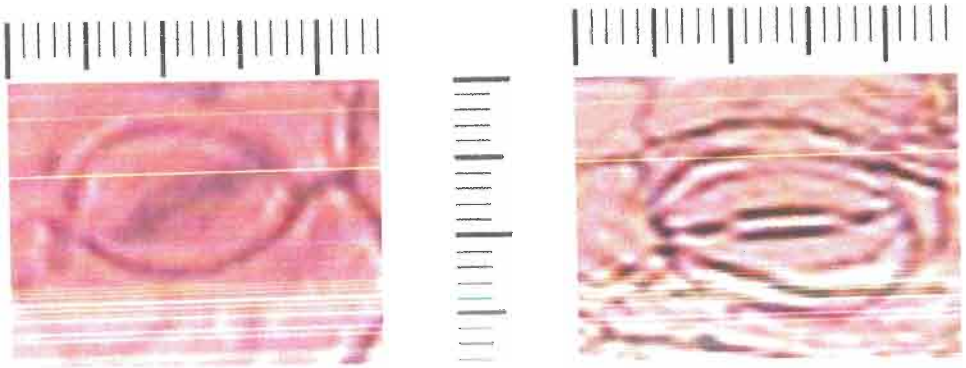
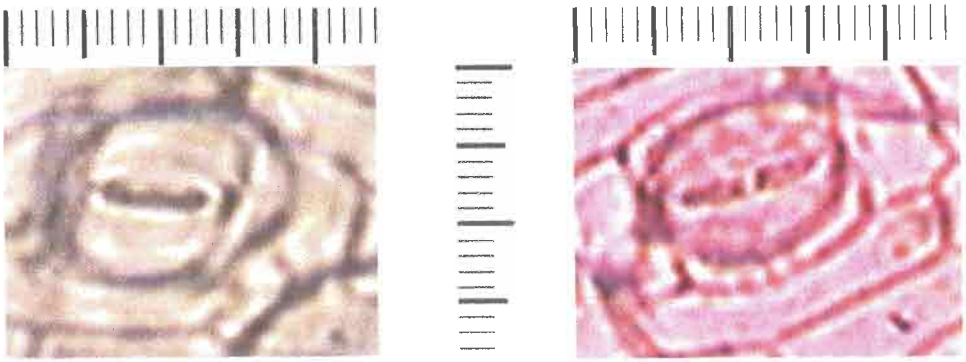


Plate 20. Size of stomata in induced tetraploid regenerants (1 division = 2.45μ)



cv.Sannachenkadali

cv.Anaikomban



cv.Kunnan

cv.Thattillakunnan

Plate 21.Size of stomata in diploid (control)
regenerants (1 division = 2.45µ)

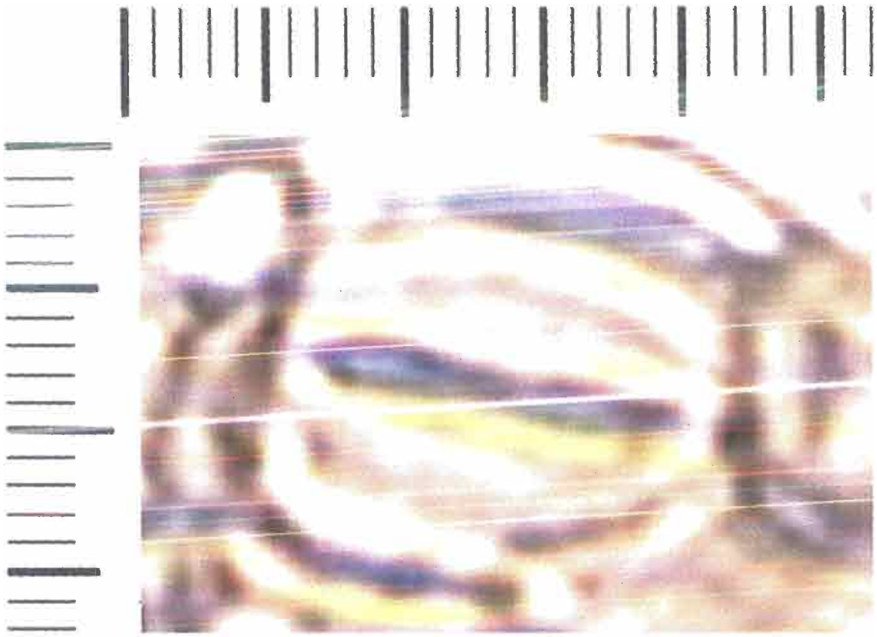


Plate 22.Size of stomata in an octoploid regenerant
(1 division = 2.45μ)

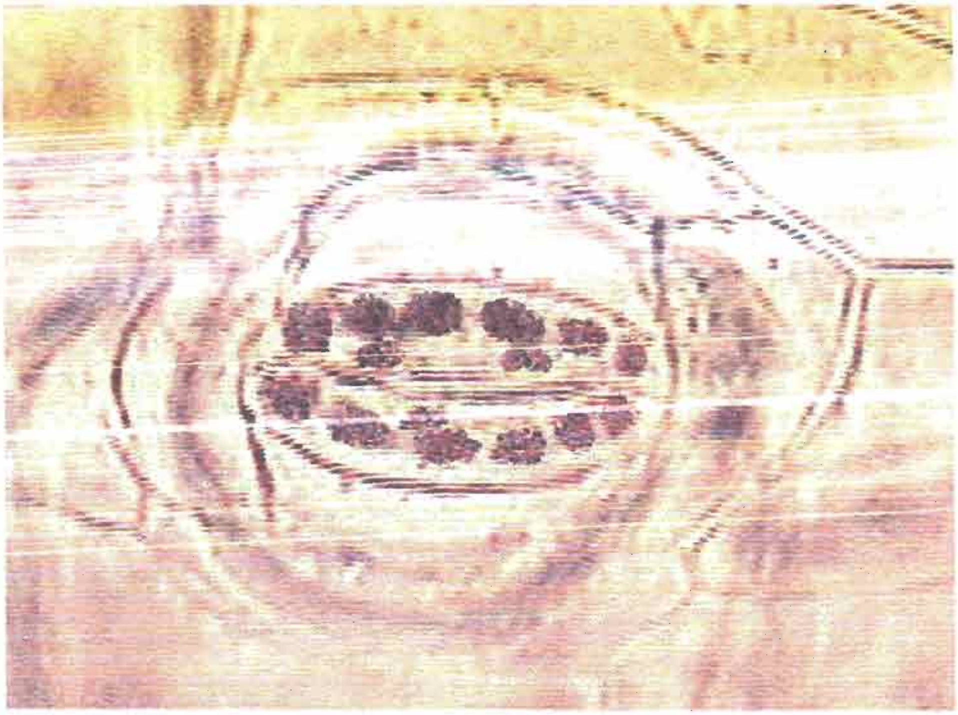


Plate 23. Chloroplast density in guard cell pair of the stomata of an induced tetraploid regenerant of the cv.Sannachenkadali

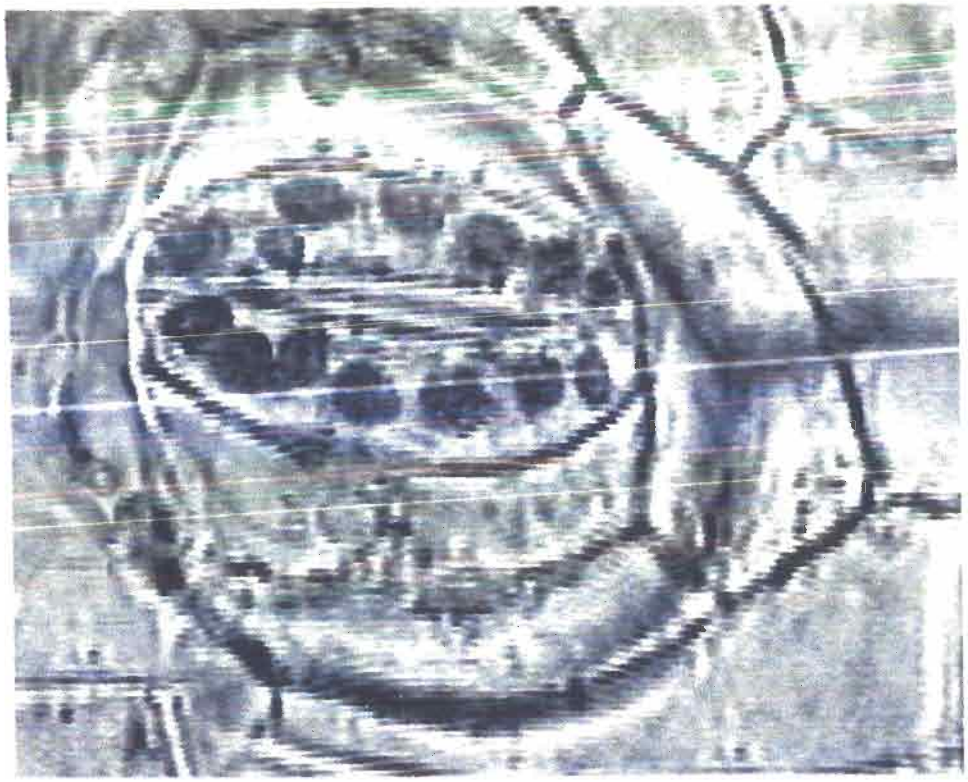
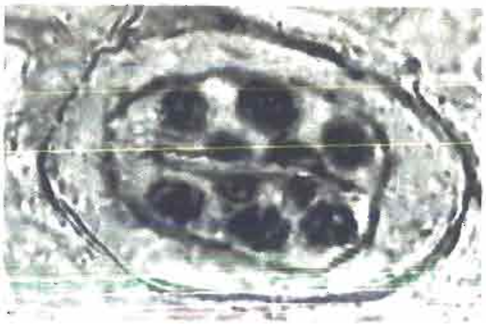


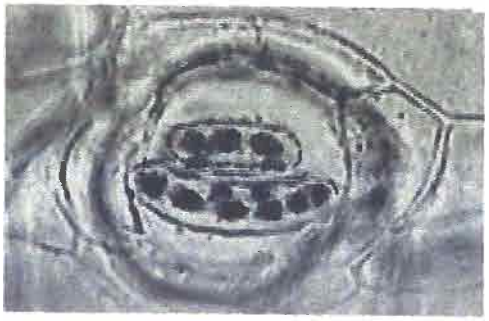
Plate 24. Chloroplast density in guard cell pair of the stomata of an induced tetraploid regenerant of the cv.Kunnan

Plate25. Chloroplast density in guard cell pair of stomata



Control (Diploid)

Octoploid



Mixoploid

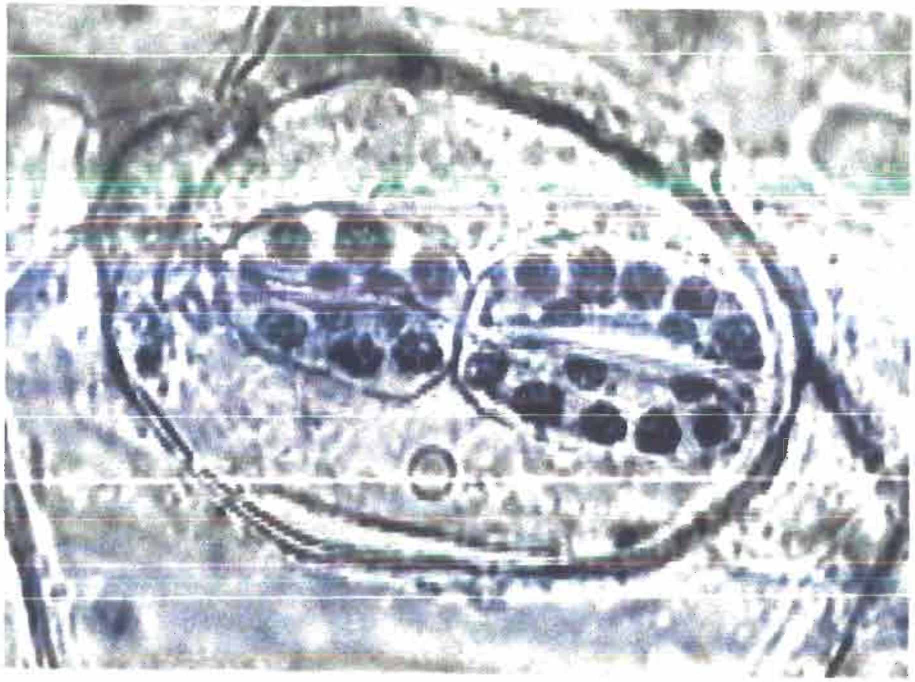


Plate 26.Chloroplast density in guard cell pair of the stomata of a mixoploid regenerant

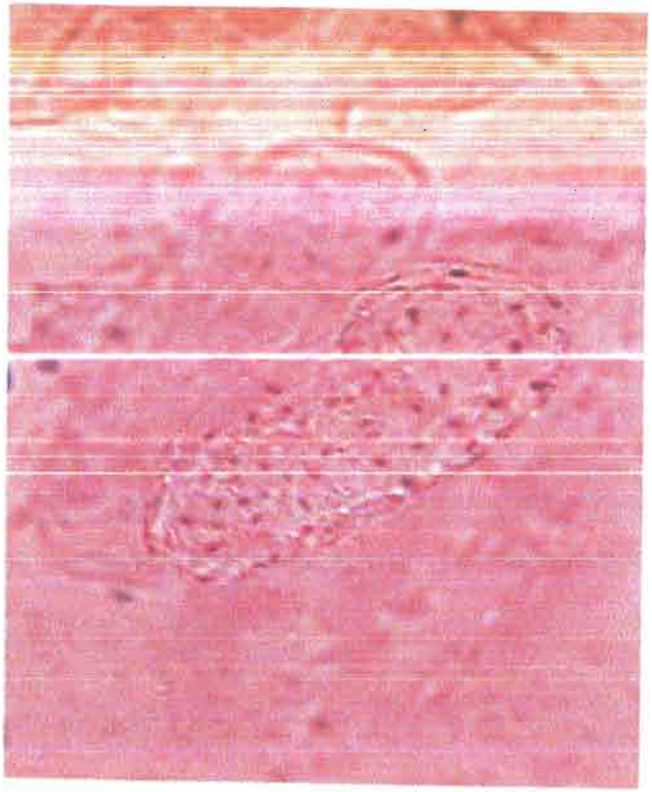


Plate 27. Root tip cells of a tetraploid (2n=4x=44) regenerant derived from colchicine-treated culture of the cv.Sannachenkadali

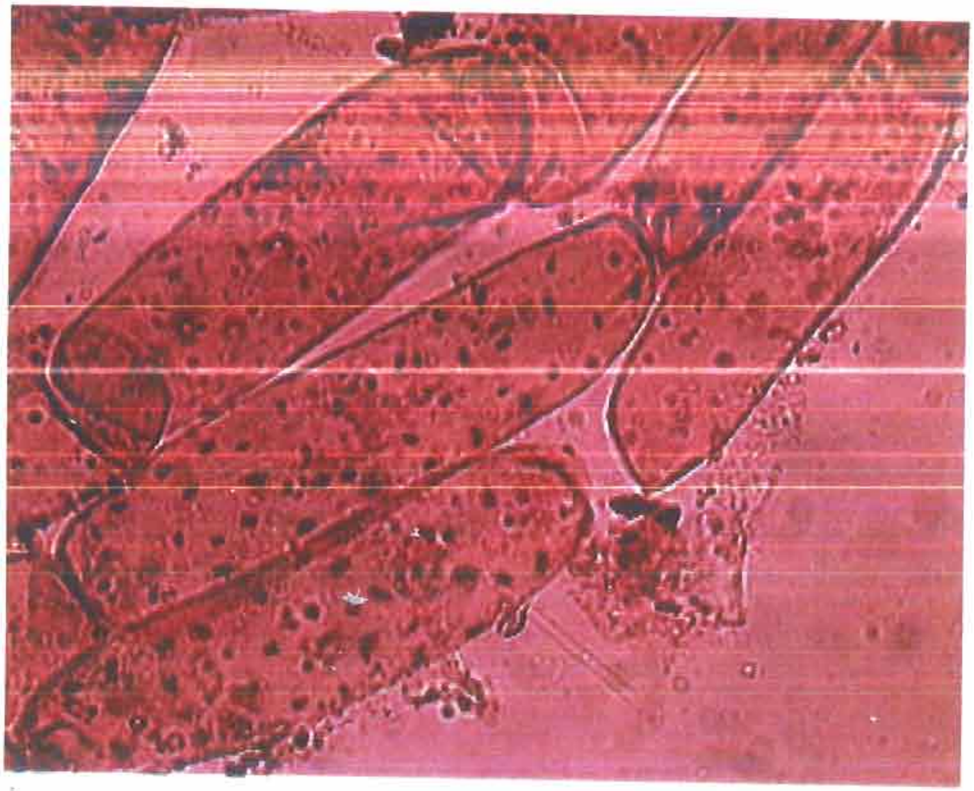


Plate 28. Root tip cells of a tetraploid (2n=4x=44) regenerant derived from colchicine-treated culture of the cv. Kunnan

Plate 29. Root tip cells of a tetraploid (2n=4x=44) regenerant derived from oryzalin-treated culture of the cv. Anaikomban

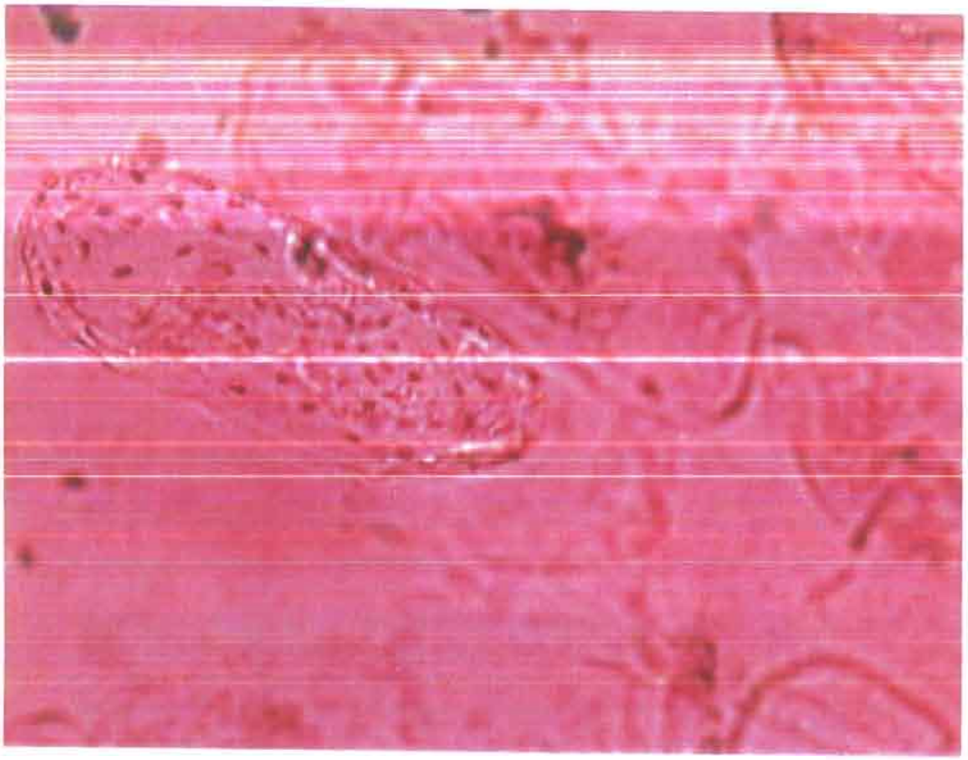
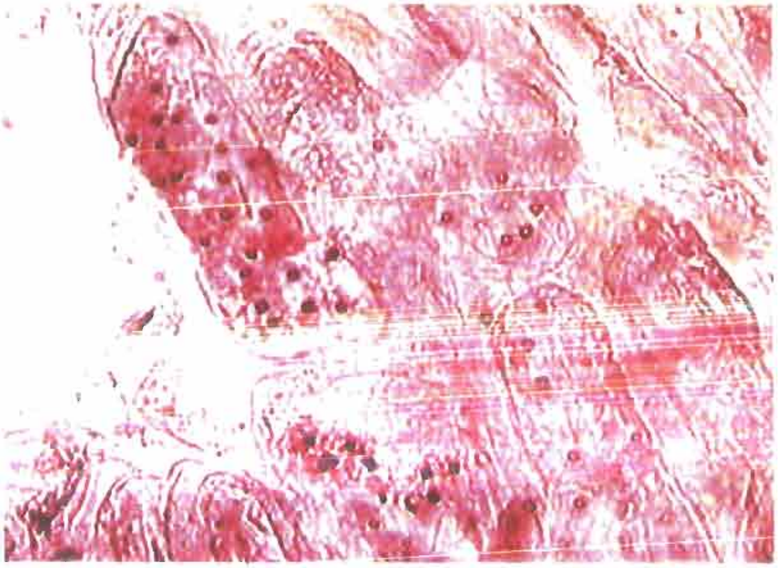


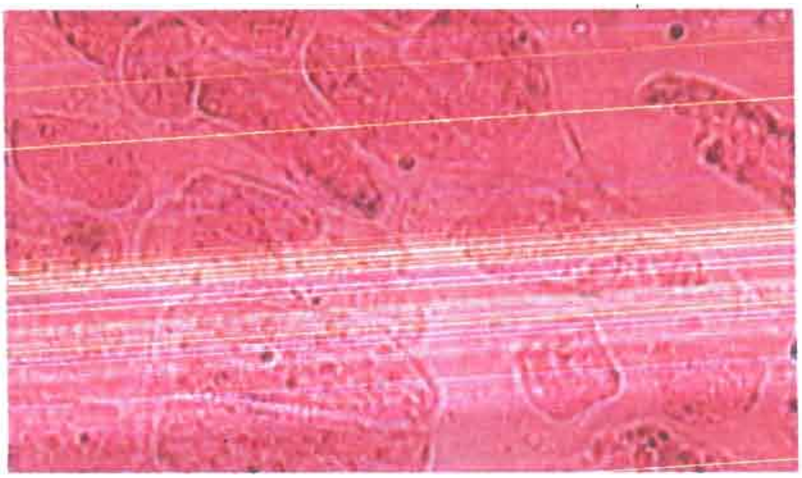
Plate 30. Root tip cells of a tetraploid (2n=4x=44) regenerant derived from oryzalin-treated culture of the cv. Thattillakunnan



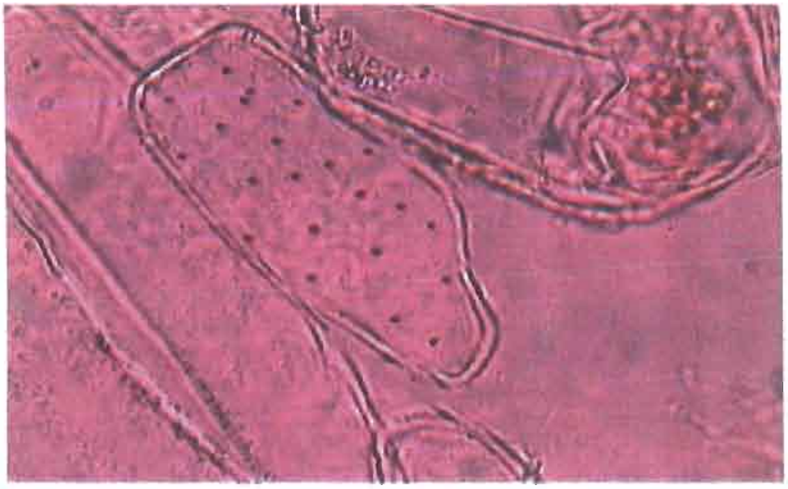
cv. Anaikomban

cv. Sannachenkadali

Plate 31. Root tip cells of diploid regenerants (2n=2x=22)

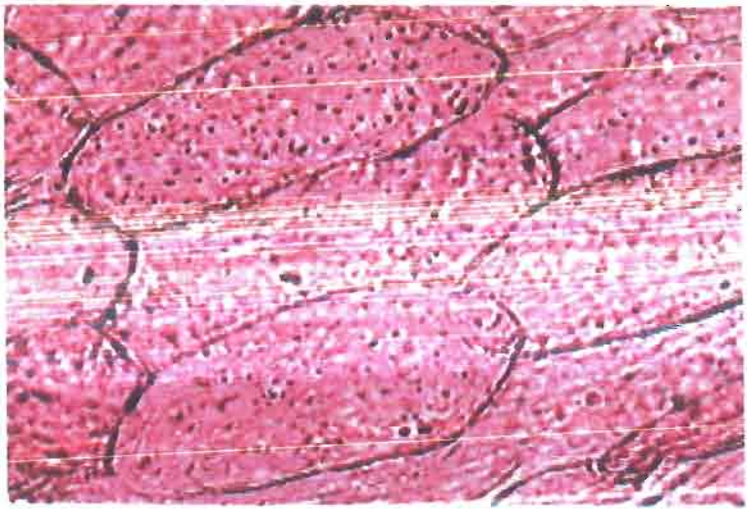


cv. Kunnan



cv. Thattillakunnan

Plate 32. Root tip cells of diploid regenerants (2n=2x=22)



cv. Sannachenkadali

cv. Kunnan

Plate 33. Root tip cells of octoploid regenerants ($2n=8x=88$)

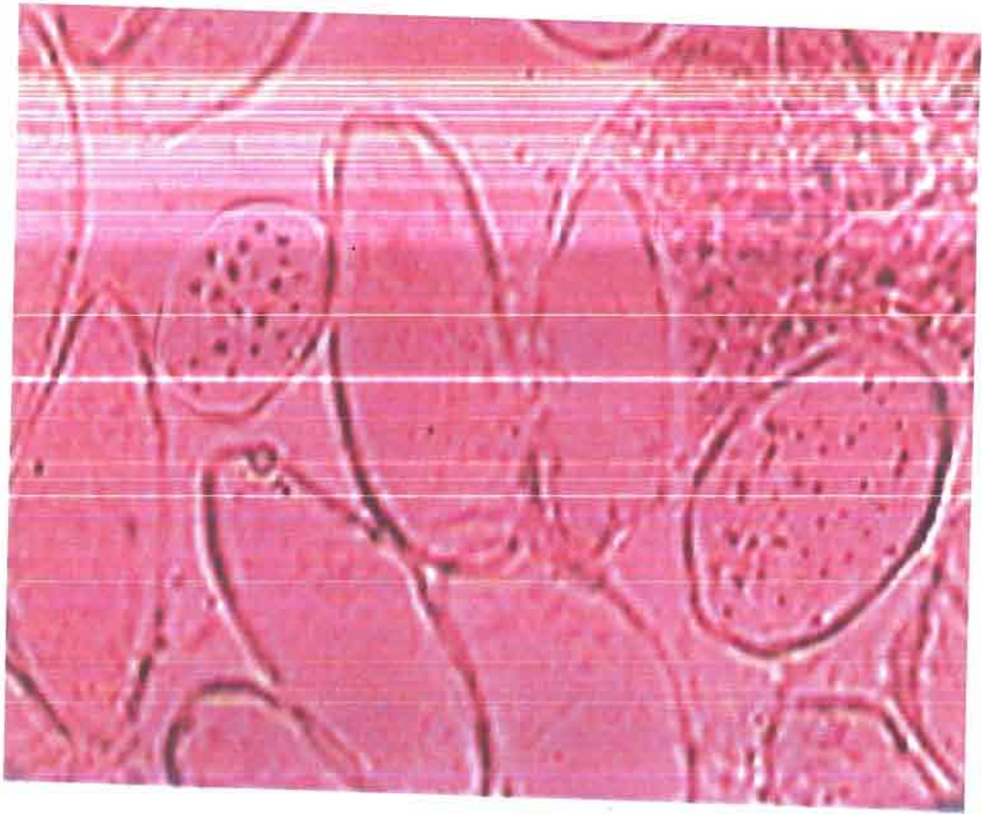
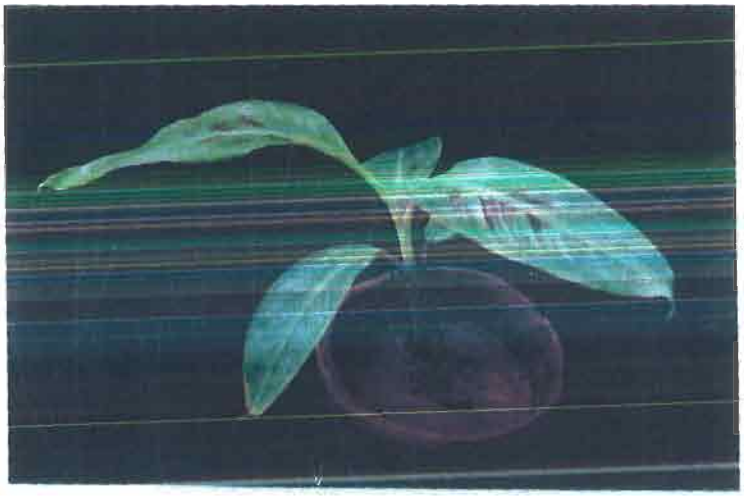


Plate 34. Root tip cells of a mixoploid regenerant (2n=2x=22 and 2n=4x=44)

Plate 35. *In vitro* induced tetraploid of the cv. Sannachenkadali



Control (Diploid)

Induced tetraploid

Plate 36. *In vitro* induced tetraploid of the cv. Anaikomban

Control (Diploid)

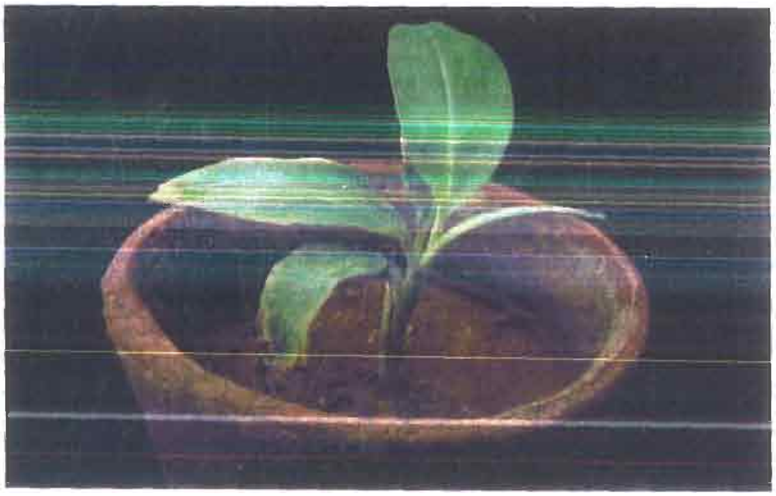
Induced tetraploid

Plate 37. *In vitro* induced tetraploid of the cv. Kunnan

Control (Diploid)

Induced tetraploid

Plate 38. *In vitro* induced tetraploid of the cv. Thattillakunnan



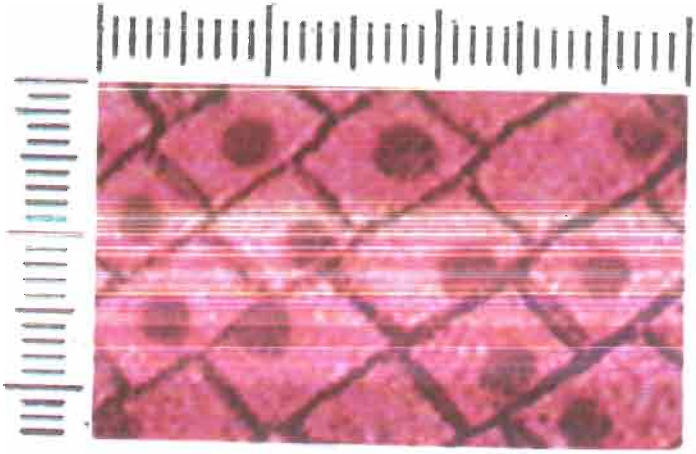
Control (Diploid)

Induced tetraploid

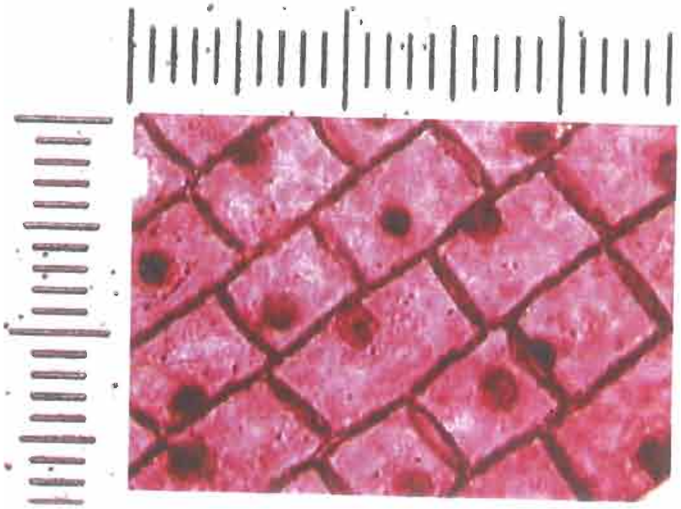
Plate 39. Anthocyanin pigmentation in the cv. Sannachenkadali

Diploid

Induced tetraploid



Induced tetraploid



Control (Diploid)

Plate 40. Interphase Nuclear Volume

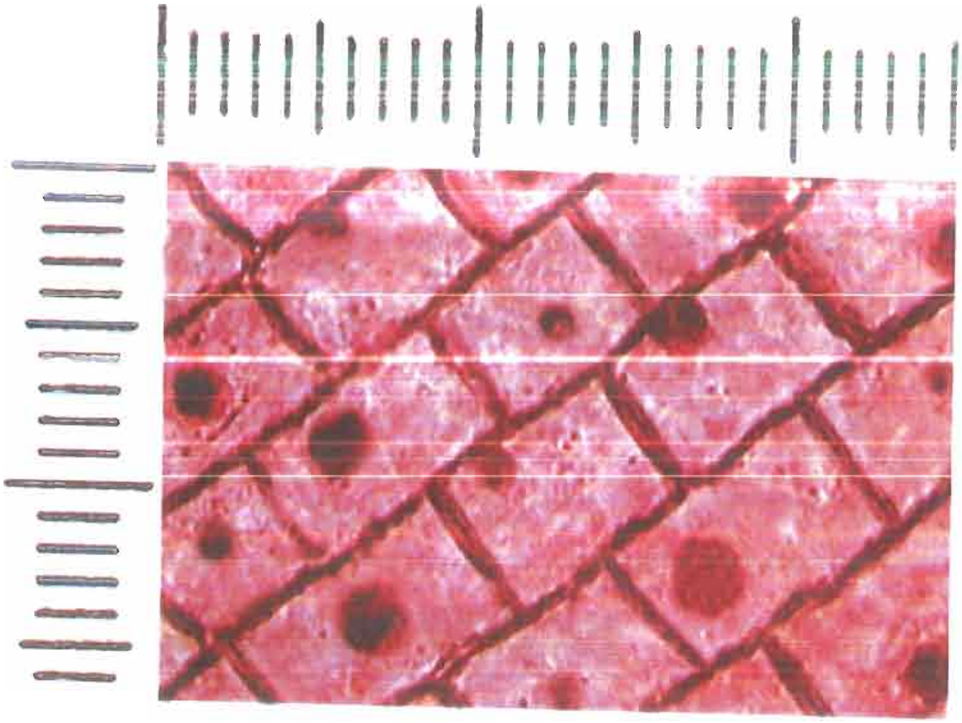


Plate 41. Interphase Nuclear Volume in a mixoploid regenerant

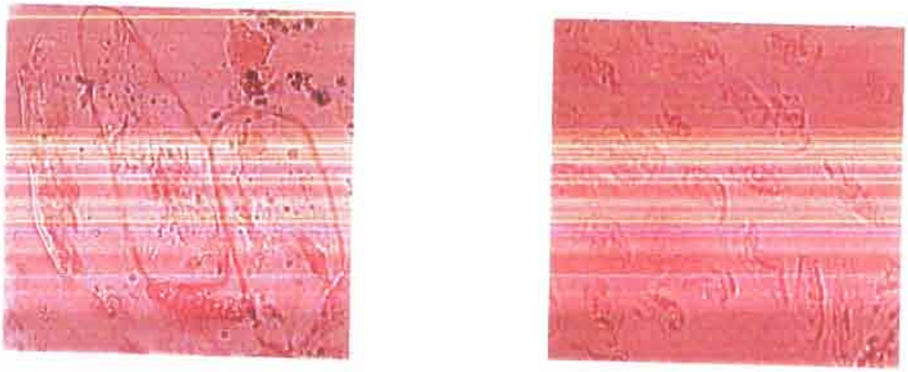
Plate42. Growth retardation in colchicine-treated cultures

C3 (7.5mM for 12h)

C4 (2.5mM for 24h)

C5 (2.5mM for 36h)

Plate43. Increase in cell size



Colchicine treatment

Control

Plate44. Multinucleate condition

Plate 45. Extrusion of cellular components

Plate 46. Excessive accumulation of cellular components

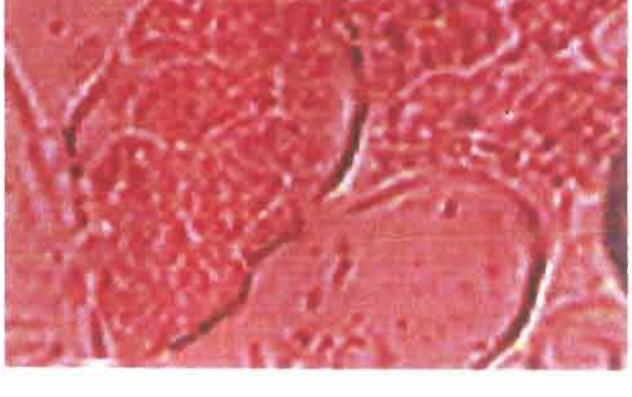
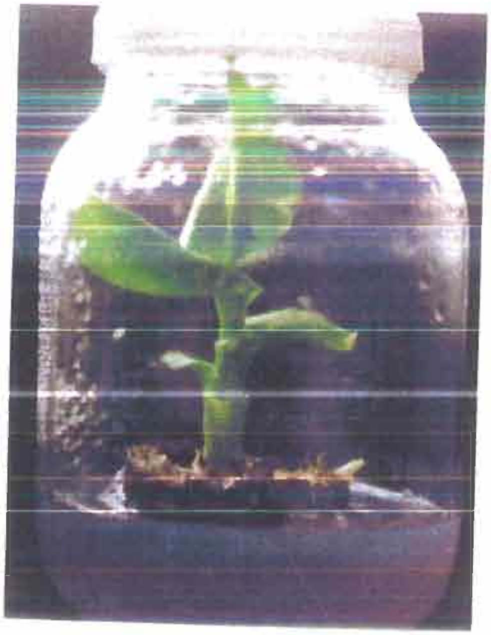


Plate 47. *In vitro* induced tetraploids with thicker leaves

Sannachenkadali-derived tetraploid

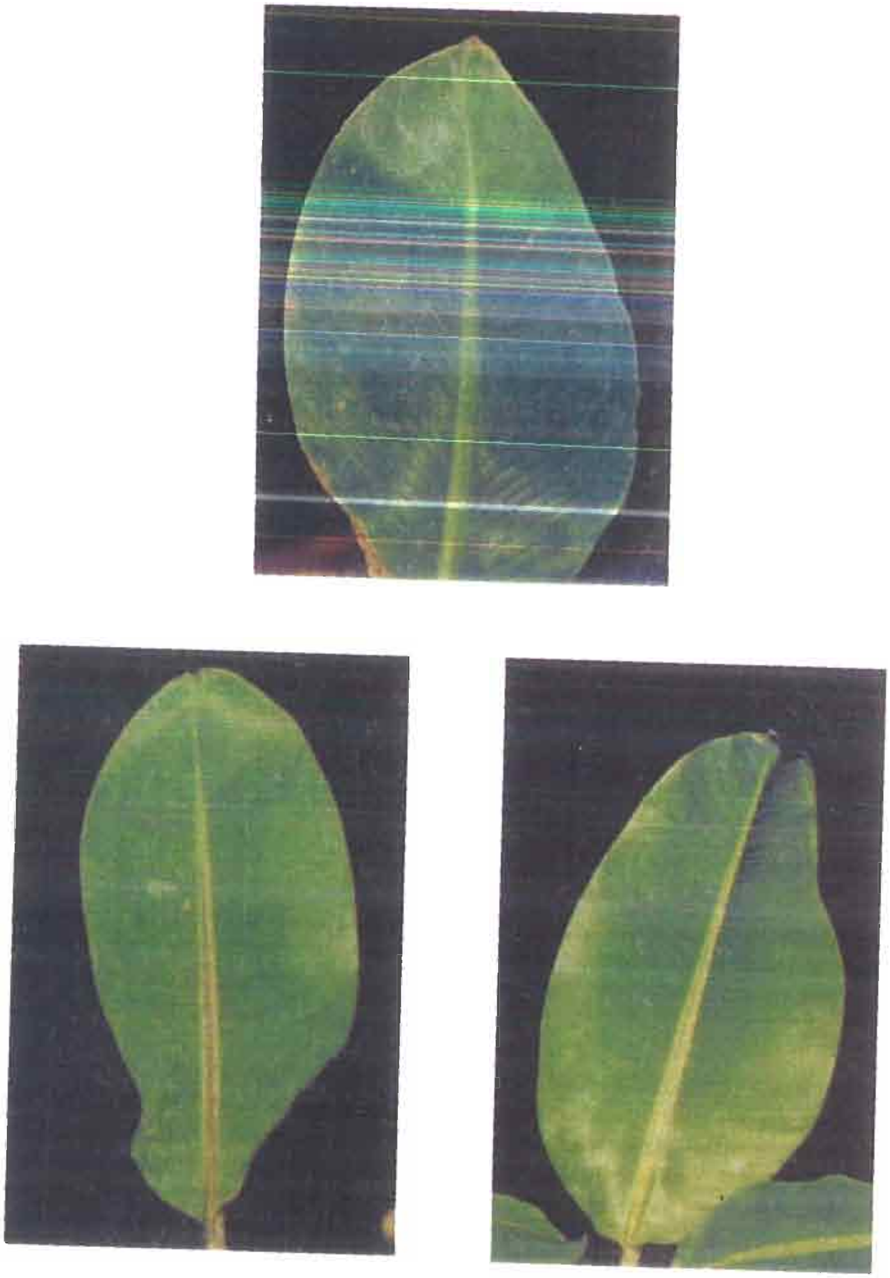


Anaikomban-derived tetraploid

Kunnan-derived tetraploid

Thattillakunnan-derived tetraploid

Plate 48. Ununiform lamina development in induced tetraploids



SUMMARY

CHAPTER VI

SUMMARY

In vitro investigations were carried out at the Tissue Culture Laboratory of the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore with the objective of assessing the feasibility of inducing tetraploids employing anti-mitotic agents in four diploid clones of banana. The clones were Sannachenkadali (AA), Anaikomban (AA), Kunnan (AB) and Thattillakunnan (AB). The anti-mitotic agents involved were colchicine and oryzalin.

Shoot tip explants of the diploid cultivars were subjected to treatment with the anti-mitotic agents under *in vitro* conditions. They were then propagated for three vegetative cycles on Murashige and Skoog (1962) medium fortified with the required growth regulators. The impact of the anti-mitotic agents on *in vitro* regeneration was assessed. The microshoots regenerated were rooted and transferred to *ex vitro* environment and the ploidy status of the regenerants were verified based on stomata and chloroplast characteristics and chromosome counts. Finally, the effect of ploidy upon various characters was analysed. Results of the investigations are summarized below.

1) The two anti-mitotic agents were associated with a delay in the response to *in vitro* regeneration, in terms of greening of shoot tip, emergence of first leaf and initiation of roots. The delay caused was greater with colchicine than with oryzalin. The earliest greening of shoot tip (7.20d after inoculation), emergence of first leaf (17.23d after inoculation) and initiation of roots (21.96d after transfer to rooting medium) was recorded by the cultures treated with O1 (10μM for 3d) which involved the

lowest concentration of oryzalin for the shortest duration. The most delayed response to the above three parameters (26.90d and 56.48d after inoculation and 44.78d after transfer to rooting medium respectively) was recorded by the treatment C5 (2.5mM for 36h) which involved the most prolonged exposure to colchicine.

2) Among the cultivars, Sannachenkadali took the least duration for shoot tip greening (9.14d after inoculation) and emergence of first leaf (21.80d after inoculation). The cultivar Anaikomban took the least duration (23.74d after transfer to rooting medium) for initiation of roots. Kunnan recorded the most delayed response to shoot tip greening (15.36d), while Thattillakunnan recorded the most delayed leaf emergence (36.46d) and root initiation (36.53d).
3) The suppression caused in the rate of response to multiple shoot regeneration in the primary culture and the three vegetative cycles was greater with colchicine than with oryzalin. The highest response in the all the stages (39.91%, 49.43%, 71.22% and 78.79% in the primary culture phase and the first, second and third vegetative cycles respectively) was recorded by the treatment O1 (10μM oryzalin for 3d). The lowest percentage of response in the primary culture phase (3.12), the first (5.60), second (6.34) and third (8.15) vegetative cycles was recorded by the treatment C5 (2.5mM for 36h), which was the most prolonged colchicine treatment.
4) Among the cultivars, the highest percentage of response to multiple shoot regeneration was recorded by Anaikomban (40.68) in the primary culture phase and Sannachenkadali in the first (51.86), second (69.03) and third (75.95) vegetative cycles. The cultivars Kunnan and Thattillakunnan recorded

lower rates of response to multiple shoot regeneration at all stages.

5) Colchicine caused a decline in the number of multiple shoots regenerated per culture, whereas oryzalin caused an increase in the same. The treatments involving lower concentrations of oryzalin regenerated more number of shoots than the untreated control. The lowest number of microshoots at all stages was regenerated by the treatment C5 (2.5mM for 36h).
6) Among the cultivars, Sannachenkadali regenerated the highest number of shoots per culture in the primary culture phase (4.67), the second (6.83) and the third (8.95) vegetative cycles and Anaikomban in the first vegetative cycle (5.22). At all stages, the cultivars Kunnan and Thattillakunnan regenerated the lower number of shoots per culture.
7) Both the anti-mitotic agents caused a suppression in the elongation of microshoots, the degree of suppression being higher with colchicine than with oryzalin. The treatment O1 (10μM for 3d) resulted in regeneration of the longest microshoots (9.35cm), while C5 (2.5mM for 36h) produced the shortest (1.95cm). Among the cultivars, Kunnan regenerated the longest mocroshoots (7.69cm), while Anaikomban regenerated the shortest (4.36cm).
8) The degree of decline caused in the weight of the regenerated microshoots was higher with colchicine than with oryzalin. The treatment O1 (10μM for 3d) produced the heaviest shoots (45.06g), while C5 (2.5mM for 36h) produced shoots with the lowest weight (15.31g). Among the cultivars, Sannachenkadali regenerated the heaviest microshoots (42.42g), while Kunnan regenerated shoots with the lowest weight (27.45g).
9) The treatment O1 (10μM for 3d) resulted in production of the highest number of leaves per plantlet (5.80) and C5 (2.5mM for

36h) the lowest (1.91). The cultivar Kunnan produced the highest number of leaves per plantlet (5.36), while Sannachenkadali produced the lowest (3.38).

10) The degree of suppression caused in the response to rhizogenesis in terms of per cent response to rooting, time taken for rooting, number of roots and length of roots was higher with colchicine than with oryzalin. The treatment O1 (10μM for 3d) recorded the highest response to rhizogenesis (65.57%), the earliest root initiation (21.96d), the highest number of roots per culture (7.77) and the longest roots (7.47cm), while C5 (2.5mM for 36h) resulted in the lowest response (6.48%), the most delayed root initiation (44.78d), the lowest number of roots per culture (0.40) and the shortest roots (0.89cm).
11) Among the cultivars, Sannachenkadali recorded the highest response (67.33%) to rooting and Kunnan the lowest (29.71%). The cultivar Anaikomban recorded the earliest root initiation (23.74d after transfer to rooting medium), while Thattillakunnan recorded the most delayed response (36.53d). The highest number of roots per plantlet (6.65) and the longest roots (6.63cm) were regenerated by the cultures of the cultivar Sannachenkadali, while the lowest number of roots (2.81) and the shortest roots (4.11cm) were produced by those of Thattillakunnan.
12) The treatment O1 (10μM for 3d) took the lowest number of days (138.19d), while C5 (2.5mM for 36h) took the highest number of days (200.26d) to complete the *in vitro* phase. Cultures of the cultivar Anaikomban underwent the shortest *in vitro* phase (144.71d), while those of Thattillakunnan underwent the longest phase (171.98d).
13) Plantlets regenerated from the cultures treated with O1 (10μM for 3d) recorded the highest *ex vitro* survival rate (73.20%) and

those regenerated from cultures treated with C5 (2.5mM for 36h) recorded the lowest (8.99%), two weeks after transfer to *ex vitro* environment. Regenerants derived from colchicine- and oryzalin-treated cultures of the four cultivars recorded *ex vitro* survival rates ranging between 38.09% and 38.65% and between 72.23% and 75.18% respectively.

14) Plantlets derived from cultures treated with O1 (10μM for 3d) recorded the highest survival rate (67.84%), while those regenerated from cultures treated with C5 (2.5mM for 36h) recorded the lowest survival rate (1.57%), two months after transfer to *ex vitro* environment. Regenerants derived from the cultivar Sannachenkadali recorded the highest survival rate (69.03%) and those derived from Thattillakunnan recorded the lowest (29.09%).

15) Ploidy assessment based on stomata analyses was 82.19% reliable, while that based on chloroplast analyses was 95.89% reliable.

16) The chromosome doubling capacity of oryzalin was higher than that of colchicine. Among the colchicine treatments, C3 induced the highest rate of tetraploidy (17.09%) and among the oryzalin treatments O4 (40μM for 3d) induced the highest rate (26.53%). Colchicine treatments resulted in induction of 15.31%, 16.11%, 10.90% and 11.13% tetraploidy in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively. Oryzalin treatments induced 20.19%, 17.19%, 17.20% and 15.89% tetraploidy in the four cultivars respectively.

17) The rates of 'other' ploidy induced by colchicine treatments were higher than those induced by oryzalin treatments. The treatment C5 induced the highest rate of 'other' ploidy (82.46%), while O1 resulted in nil 'other' ploidy.

18) Colchicine treatments resulted in induction of 46.12%, 34.94%, 59.88% and 53.30% of 'other' ploidy in the cultivars Sannachenkadali, Anaikomban, Kunnan and Thattillakunnan respectively. Oryzalin treatments induced 9.66%, 9.87%, 14.41% and 20.76% 'other' ploidy in the four cultivars respectively.
19) At the end of the hardening phase and two months after transfer to *ex vitro* environment, the pseudostem height and girth of the induced tetraploids remained lower than those of their corresponding diploids. At four months after transfer, the pseudostem height and girth of the induced tetraploids had increased and had surpassed the corresponding diploids. The leaf area of the induced tetraploids was higher than that of the corresponding diploids.
20) The phyllochron values of the induced tetraploids ranged between 14.00 and 17.00d, 13.00 and 15.00d and 7.00 and 10.00d at transfer to *ex vitro* environment and after two months and four months of transfer respectively.
21) The induced tetraploids had lower leaf LWR's, heavier leaves and lower petiole dry weight percentages compared to their respective diploids.
22) The induced tetraploids of the four cultivars had higher chlorophyll contents than their corresponding diploids. Tetraploids derived from the cultivar Sannachenkadali had higher anthocyanin contents than the corresponding diploid plants.
23) The induced tetraploids recorded higher photosynthetic efficiencies than the control plants. The crop growth rates were initially lower and at later stages increased above those of the corresponding diploid plants.

24) The INV's of the induced tetraploids were higher than those of the corresponding diploids.

The induced tetraploids are now in their vegetative phase. Assessment of their genomic constitution, fertility status, level of disease resistance, economic traits, crossability with diploids etc., will be taken up in the future and the potential tetraploids may be used in future breeding programmes.

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*Originals not seen

ABBREVIATIONS

BAP	Benzyl amino purine
GA_3	Gibberellic acid
NAA	Naphthalene acetic acid
IBA	Indole butyric acid
DMSO	Dimethyl sulfoxide
V_1	First vegetative cycle
V_2	Second vegetative cycle
V_3	Third vegetative cycle
MS	Murashige and Skoog (1962) medium
LWR	Length-width ratio
INV	Interphase nuclear volume
DNH	Dinitroaniline herbicides
S-Tetra	Sannachenkadali-derived induced tetraploid
A-Tetra	Anaikomban-derived induced tetraploid
K-Tetra	Kunnan-derived induced tetraploid
T-Tetra	Thattillakunnan-derived induced tetraploid
GCP	Guard cell pair
AMP	Amiprophos methyl
h	Hours
d	Days

APPENDICES

Appendix 1. Composition of MS (Murashige and Skoog, 1962) medium

Component	Requirement for 1litre of medium
Macronutrients	**mg l^{-1}**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	**mg l^{-1}**
KI	0.830
H_3BO_3	6.200
$MnSO_4.4H_2O$	22.300
$ZnSO_4.7H_2O$	8.600
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	0.025
$FeSO_4.4H_2O$	27.800
Na_2.EDTA	37.300
Vitamins and organics	
myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Sucrose (gl^{-1})	30
pH	5.8

Appendix 2. Details of colchicine treatments

S.No.	Concentration of colchicine (mM)	Duration of treatment (h)
1	2.5	12
2	5.0	12
3	7.5	12
4	10.00	12
5	2.5	24
6	5.0	24
7	7.5	24
8	10.00	24
9	2.5	36
10	5.0	36
11	7.5	36
12	10.00	36
Control	-	-

Appendix 3. Details of oryzalin treatments

S.No.	Concentration of oryzalin (μM)	Duration of treatment (d)
1	10.00	3
2	20.00	3
3	30.00	3
4	40.00	3
5	50.00	3
6	10.00	6
7	20.00	6
8	30.00	6
9	40.00	6
10	50.00	6
Control	-	-

Appendix 4. Effect of colchicine on the survival rates of shoot tip cultures

Treatment No.	Treatment		% survival				Mean
	Conc. of colchicine (mM)	Treatment duration (h)	S	A	K	T	
T0	Control		100.00	100.00	100.00	100.00	100.00
T1	2.5	12	56.12 (48.52)	61.23 (51.49)	62.77 (52.40)	57.00 (49.03)	59.28
T2	5.0	12	54.24 (47.43)	53.21 (46.84)	51.20 (45.69)	53.41 (46.96)	53.02
T3	7.5	12	52.664(6.53)	50.22 (45.13)	48.19 (43.96)	50.18 (45.10)	50.31
T4	10.0	12	41.46 (40.08)	41.23 (39.95)	41.18 (39.92)	39.55 (38.97)	40.86
T5	2.5	24	54.14 (47.38)	52.31 (46.32)	50.00 (45.00)	50.00 (45.00)	51.61
T6	5.0	24	33.10 (35.12)	34.17 (35.77)	30.19 (33.33)	30.19 (33.33)	31.91
T7	7.5	24	27.33 (31.52)	26.21 (30.80)	24.16 (29.44)	25.14 (30.09)	25.71
T8	10.0	24	12.18 (20.43)	14.16 (22.11)	12.99 (21.13)	11.23 (19.58)	12.64
T9	2.5	36	48.21 (43.98)	50.29 (45.77)	48.23 (43.99)	48.16 (43.95)	48.72
T10	5.0	36	26.88 (31.23)	27.00 (31.31)	25.44 (30.29)	26.11 (30.73)	26.36
T11	7.5	36	8.31 (16.75)	7.26 (15.63)	7.41 (15.80)	7.63 (16.04)	7.65
T12	10.0	36	0.00. (0.573)	2.08 (8.29)	0.00 0(.573)	1.92 (7.96)	2.00

Numbers in parantheses are arc-sine transformed values

Appendix 5. Effect of oryzalin on the survival rates of shoot tip cultures

Treatment No.	Treatment		% survival				Mean
	Conc. of oryzalin (µM)	Treatment duration (d)	SCK	ANK	K	TK	
T0	Control		100.00	100.00	100.00	100.00	100.00
T1	10.00	3	83.00 (65.65)	86.25 (68.24)	79.62 (63.17)	84.36 (66.71)	83.31
T2	20.00	3	75.00 (60.00)	72.27 (58.23)	73.33 (58.91)	72.14 (58.14)	73.19
T3	30.00	3	58.33 (49.80)	60.42 (51.02)	60.00 (50.77)	58.33 (49.80)	59.27
T4	40.00	3	53.33 (46.91)	56.25 (48.59)	53.33 (46.91)	52.65 (46.52)	58.02
T5	50.00	3	42.33 (40.59)	42.35 (40.60)	33.33 (35.26)	40.00 (39.23)	39.50
T6	10.00	6	68.36 (55.77)	53.33 (46.91)	50.00 (45.00)	50.00 (45.00)	55.42
T7	20.00	6	53.33 (46.91)	50.00 (45.00)	48.65 (44.23)	43.45 (41.24)	48.86
T8	30.00	6	33.33 (35.26)	31.25 (33.99)	29.16 (32.68)	29.78 (33.07)	30.88
T9	40.00	6	27.08 (31.36)	25.00 (30.00)	26.67 (31.09)	25.00 (30.00)	25.94
T10	50.00	6	13.33 (21.41)	13.33 (21.41)	12.56 (20.76)	13.33 (21.41)	3.14

Numbers in parantheses are arc-sine transformed values