PARASEXUAL HYBRIDIZATION OF *Piper nigrum* AND *Piper colubrinum* THROUGH PROTOPLAST FUSION

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

Faculty of Agriculture

Kerala Agricultural University

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COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA

2000

DECLARATION

I hereby declare that this thesis entitled "**Parasexual hybridization of *Piper nigrum* and *Piper colubrinum* through protoplast fusion**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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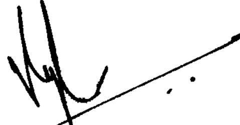

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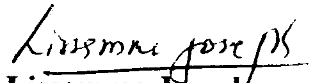

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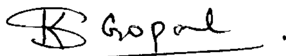

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ACKNOWLEDGEMENT

"Unto Him who is everything, I owe everything"

I express my deepest sense of gratitude and indebtedness to **Dr.P.C.Rajendran**, Associate Professor, Department of Plantation Crops and Spices and Chairperson of my advisory committee for his inspiring guidance, timely and valuable suggestions and consistent encouragement throughout the course of this investigation.

It is out of my knowledge to express in words my deepest sense of gratitude to **Smt.Lisamma Joseph**, Assistant Professor, Department of Plantation Crops and Spices for her everwilling help, valuable advise, incessant patience, scholarly suggestions, kind concern and unstinting support rendered at all stages of work.

With deep respect, I place my thanks to **Dr.E.V.Nybe**, Associate Professor and Head, Department of Plantation Crops and Spices for valuable and timely help rendered during the course of research.

My heartfelt thanks are due to **Dr.K.Surendragopal,** Assistant Professor, Department of Plant Pathology. His critical comments indeed helped me a lot in improving the text.

I am extremely grateful to each and every member of the Department of Plantation Crops and Spices for extending all possible help in the proper conduct of the research work.

I wish to express and place on my record my heartfelt thanks to Dr.V.K.Mallika, Associate Professor, CCRP for the photographs taken for the thesis.

My sincere thanks are also due to Dr.P.A.Nazeem, Associate Professor, CPBMB for providing the lab facilities needed for the smooth conduct of the study.

May I also take this opportunity to thank Dr.K.V.Sureshbabu and Sri.S.Krishnan who showed ardent interest and gave inspiring encouragement at different periods of the study.

Award of Junior Fellowship of Kerala Agricultural University is gratefully acknowledged.

I would like to record my special and heartfelt gratitude to Thara, Prajitha, Anil, Rajesh, Achuthan and Babu, Research Associates at CPBMB for their help during the course of investigation.

My sincere thanks are also due to my friends Kavitha, Lekshmy, Samasya, Parvathy, Viji, Sanchu, Manjusha, Sreeja, Annie, Reeni chechy, Shirish, Govind and Niranjan for their help and constant encouragement.

The help rendered for the photographic works by Mr.Sreekumar is gratefully acknowledged.

I thank all the labourers of the Tissue Culture laboratory for their help.

My appreciation also goes to Mr.Joy and family for the neat typing of the manuscript.

Valsachechi is another person to whom I will be indebted. My obligation to chechi and her family cannot be expressed in words.

I am forever beholden to my Pappa, Mummy, Kuttan, Appachan and Amma for their boundless affection, care, encouragement, support and prayers.

Resmi Paul

Dedicated to my loving
Pappa and Mummy

CONTENTS

Chapter	Title	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	4
3	MATERIALS AND METHODS	24
4	RESULTS	32
5	DISCUSSION	51
6	SUMMARY	60
	REFERENCES	
	APPENDICES	
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1	Enzymes tried for protoplast isolation from *in vitro* leaves of *Piper nigrum*	27
2	Enzymes tried for protoplast isolation from *in vitro* leaves of *Piper colubrinum*	28
3	Levels of osmoticum tried for protplast isolation	28
4	Influence of varying duration of incubation and osmotic potential on protoplast yield ($x10^4$) of *Piper nigrum* with E_1	33
5	Influence of varying duration of incubation and osmotic potential on protoplast viability (%) of *Piper nigrum* with E_1	33
6	Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper nigrum* at 0.6 M	36
7	Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper nigrum* at 0.6 M	36
8	Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper nigrum* at 0.65 M	36
9	Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper nigrum* at 0.65 M	39
10	Influence of different centrifugal speed and duration on protoplast yield ($x10^4$) of *Piper nigrum*	39
11	Influence of density gradient on protoplast yield ($x10^4$) of *Piper nigrum*	39
12	Influence of varying duration of incubation and osmotic potential on protoplast yield ($x10^4$) of *Piper colubrinum* with T_1	41

13	Influence of varying duration of incubation and osmotic potential on protoplast viability (%) of *Piper colubrinum* with T_1	41
14	Influence of varying duration of incubation on protoplast yield ($x10^4$) of *Piper colubrinum* with T_2	44
15	Influence of varying duration of incubation on protoplast viability (%)of *Piper colubrinum* with T_2	44
16	Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper colubrinum* at 0.6 M	44
17	Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper colubrinum* at 0.6 M	47
18	Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper colubrinum* at 0.65 M	47
19	Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper colubrinum* at 0.65 M	47
20	Influence of different centrifugal speed and duration on protoplast yield ($x10^4$) of *Piper colubrinum*	49
21	Influence of density gradient on protoplast yield ($x10^4$) of *Piper colubrinum*	49
22	Survival of protoplasts during culture	49

LIST OF PLATES

Plate No.	Title
1	*In vitro* cultures of *Piper nigrum*
2	*In vitro* cultures of *Piper colubrinum*
3	Interconnections between cells of *Piper nigrum* (E_1, 15 h, 0.6 M) (100 x)
4	Isolated protoplasts of *Piper nigrum* (E_8, 21 h, 0.6 M) (200 x)
5	Isolated protoplasts of *Piper nigrum* (E_7, 21 h, 0.6 M) (50 x)
6	Interconnections between cells of *Piper colubrinum* (T_1, 15 h, 0.6 M) (100 x)
7	Isolated protoplasts of *Piper colubrinum* (T_2, 21 h, 0.6 M) (200 x)
8	Isolated protoplasts of *Piper colubrinum* (T_8, 21 h, 0.6 M) (100 x)
9	Isolated protoplasts of *Piper colubrinum* (T_2, 17 h, 0.65 M) (200 x)

LIST OF FIGURES

Fig. No.	Title
1	Influence of concentration of osmoticum in enzyme solution (E_1) on total and live protoplasts of *Piper nigrum* during 17 to 23 h
2	Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper nigrum* at 0.6 M
3	Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper nigrum* at 0.65 M
4	Influence of concentration of osmoticum in enzyme solution (T_1) on total and live protoplasts of *Piper colubrinum* during 17 to 23 h
5	Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper colubrinum* at 0.6 M
6	Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper colubrinum* at 0.65 M

LIST OF APPENDICES

Appendix No.	Title
I	Basal media for perennial crop protoplast culture
II	Chemical composition of Murashige and Skoog (1962) medium
III	Chemical composition of protoplast culture medium

ABBREVIATIONS

AR	analytical reagent
M	molar
mM	milli molar
μM	micro molar
cv.	cultivar
h	hour
ml^{-1}	per milli litre
w/v	weight by volume
g*	gravity
μm	milli micron
rpm	revolution per minute
MS	Murashige and Skoog
$mg\ l^{-1}$	milli gram per litre
BAP	benzyl amino purine
BA	benzyl adenine
IAA	indole - 3 - acetic acid
NAA	1 - naphthalene acetic acid
2, 4-D	2, 4-dichloro phenoxy acetic acid
MES	2 - (N - morpholino) ethane sulphonic acid
PEG	poly ethylene glycol
μ	micron
ppm	parts per million
g	gram
dia	diameter
ABA	abscissic acid
ml	milli litre
%	per cent
t	ton
$HgCl_2$	mercuric chloride
UV	ultra violet
psi	pounds per square inch
min	minutes
FDA	fluorescein diacetate
CPW	cell and protoplast washing solution
MW	molecular weight
NH_4^+	ammonium ion
Ca^{2+}	calcium ion
PCM	protoplast culture medium

INTRODUCTION

INTRODUCTION

Black pepper (*Piper nigrum* L.), the king of spices, popularly known as black gold is the most important export oriented commodity and foreign exchange earner among the Indian spices. In India, pepper is grown in an area of 2.38 lakh hectare with a production of 75,000 tons (Nambiar and Menon, 2000). Kerala alone accounts for more than 90 per cent of the area and production. Black pepper accounts for 47.63 per cent (Rs.479.57 crores) of the total export earning from spices in the year 1999-2000.

The Indian pepper enjoyed monopoly in the world market till the turn of 19th century, when other countries like Indonesia and Brazil entered the pepper trade. The productivity in India is the lowest when compared to other pepper producing countries such as Indonesia, Malaysia and Brazil.

The production of black pepper is dwindled in recent times due to severe crop losses caused by foot rot fungus *Phytophthora capsici*. Not only the production, but also the wealth of genetic diversity available in the crop is plagued with this dreadful disease. Many of the valuable genotypes are lost every year from the gene pool due to this serious malady.

The crop loss due to *Phytophthora* foot rot disease has been estimated by different workers. Samraj and Jose (1966) and Nambiar and Sarma (1977) reported 20-30 per cent of vine death in Kannur and Kozhikode districts. An annual loss of 905 and 119 t of black pepper respectively was reported from these districts by Balakrishnan *et al.* (1986) and Anandaraj *et al.* (1988). Similarly Sarma *et al.* (1994) estimated the annual crop loss to the tune of 4.5 to 7.5 million dollars on a global scale.

All the black pepper cultivars are susceptible to this disease. Resistance against this serious malady has not been identified in the germplasm of cultivated *Piper nigrum*. However, *Piper colubrinum* Link, a wild relative of black pepper was found immune to foot rot disease (Turner, 1973 and Sarma *et al.*, 1991). The field establishment of grafts involving *P. nigrum* and *P. colubrinum* had been reported (Albuquerque, 1968).

Biotechnological tools become relevant in this context. Molecular breeding is increasingly being used in crop improvement of several important crops. Isolated protoplasts offer an excellent experimental system for molecular, biochemical and physiological studies and can also serve as a tool for production of genetically transformed plants. Protoplasts, unlike complex explants offer the possibility for direct transformation of plants (Gunn and Day, 1986) and for the generation of somaclonal variation. Due to the absence of cell wall, plant protoplast has a remarkable property of being able to take up various organelles such as DNA, mitochondria, chloroplast and chromosomes. Moreover, they offer the possibility for somatic hybridization facilitating gene flow between species and bypassing of reproductive barriers. Somatic hybridization to transfer cytoplasmically encoded traits such as cytoplasmic male sterility and resistance to pests and diseases or direct transfer of a few nuclear genes from wild species to cultivated species offers new options that could complement conventional hybridization approaches. Several positive characters have been incorporated into many plants by protoplast fusion (Grosser *et al.*, 1992; Guo and Deng , 1998; Henn *et al.*, 1998).

Biotechnological means like somatic hybridization and gene transfer are more appropriate to develop resistant black pepper lines. The importance of protoplast technology in disease resistance has been reported by several workers (Takebe and Nagate, 1984; Panis *et al.*, 1993).

The present investigation of parasexual hybridization between *P. nigrum* and *P. colubrinum* was undertaken with the following objectives. Standardisation of methods for

i) Protoplast isolation

ii) Protoplast purification

iii) Protoplast culture

iv) Protoplast fusion between *P. nigrum* and *P. colubrinum*

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Phytophthora foot rot disease incited by *Phytophthora capsici* is an ubiquitous disease of black pepper (*Piper nigrum*) posing serious threat to its cultivation. The disease was reported in India as early as 1902 (Menon, 1949) from Wynad region of Kerala. There is no effective control measure to tackle the disease and all the cultivated types are susceptible to the disease (Sarma *et al.*, 1982). *Piper colubrinum*, a wild species of pepper introduced from Amazone river basin is reported to be immune to *Phytophthora capsici* (Turner, 1973 and Sarma *et al.*, 1991). Screening the available genetic diversity of black pepper for resistance to *Phytophthora* foot rot was done by Kueh and Khew (1980). Smith and McCown (1982) stated that techniques of parasexual hybridization may allow exchange of germplasm between sexually immature superior plants and possibly between cross-incompatible or sterile parents. When sexual crosses become prohibitive because of absolute incompatibility, production of bridge hybrid by cell fusion may be the feasible alternative (Gamborg and Nabors, 1992). Importance of protoplast technology in disease resistance has been supported by several workers (Takebe and Nagate, 1984; Panis *et al.*, 1993 and Tican and Menczel, 1998). Wang and Lorz (1994) suggested that since wild barley which is resistant to a wide range of pathogens and tolerant to environmental stress, do not hybridize with cultivated barley, modern biotechnological methods, such as somatic hybridization and transformation via direct DNA delivery must be used to obtain gene transfer. In this context, biotechnological means like somatic hybridization and gene transfer are more appropriate to develop resistant black pepper lines. An attempt has been made to review various reports on protoplast isolation, purification, culture and fusion.

2.1 Protoplast isolation

Protoplasts are cells from which the cell wall has been removed by mechanical and/or enzymatic methods (Carlson, 1973). Isolation of protoplasts can be achieved either by mechanical methods or enzymatic methods.

Mechanical isolation of protoplasts is done by cutting plasmolysed tissue with a sharp edged knife and releasing the protoplasts by deplasmolysis (Klercker, 1892). The principal deficiency of this approach is that the protoplasts released are few in number. Mechanical isolation is thus only of historical importance now.

Enzymatic method was put forward by Power and Cocking (1970). They effectively isolated protoplasts from fully expanded leaves of young tobacco plants using 2 per cent cellulase and 0.5 per cent macerozyme.

2.2 Factors affecting protoplast isolation

Factors affecting protoplast isolation include donor tissue, osmoticum, concentration of enzymes, time of incubation, pH and temperature (Evans and Bravo, 1983).

2.2.1 Donor tissue

2.2.1.1 Types of donor tissue

Protoplasts could be isolated from various tissues.

2.2.1.1.1 Leaves

Isolation of protoplasts from leaves has been suggested in *Pisum sativum* and *Hordeum vulgare* (Kao and Michayluk, 1974), *Alnus glutinosa* (Huhtinen *et al.*, 1982), *Carica papaya* (Liu and Yang, 1983), *Broussonentia kazinoki* (Oka and Ohyama, 1985), *Brassica oleraceae* (Kik and Zaal, 1993), *Actinidia* spp. (Xiao and Hirsch, 1996), *Piper nigrum* and *P. colubrinum* (Philip *et al.*, 1998), *Moricanda nitens* (Tian and Meng, 1999).

2.2.1.1.2 Callus and suspension culture

Protoplast isolation from calli and suspension cultures has been reported in *Vicia hajastana* and *Glycine max* (Kao and Michayluk, 1974), tobacco

(Uchimiya and Murashige, 1974), *Citrus aurantium, C. limon, C. paradisi* (Vardi *et al.*, 1982), *Citrus sinensis* (Kobayashi *et al.*, 1985), *Carica papaya* (Litz, 1986), *Citrus reticulata* (Hidaka and Kajiura, 1988), *Musa* spp. (Panis *et al.*, 1993), *Gossypium hirsutum* (Peeters *et al.*, 1994), *Nicotiana africana* (Tican and Menczel, 1998) and *Bupleurum falcatum* (Bang *et al.*, 1999).

2.2.1.1.3 Cotyledon

Isolation of cotyledonary protoplasts has been reported in *Pinus pinaster* (David and David, 1979), *Populus simonii* (Zhang and Liang, 1981), *Carica papaya* (Litz, 1986) and *Brassica campestris* (Zhao *et al.*, 1994).

2.2.1.1.4 Pollen

Redenbaugh *et al.* (1980) isolated *Ulmus americana* protoplasts from pollen mother cells, tetrads and microspores. Fellner and Havranek (1992) reported isolation of protoplasts from pollen grains of *Allium* spp.

2.2.1.2 Age of donor tissue

Schenk and Hildebrandt (1969) and Uchimiya and Murashige (1974) opined that younger plant parts were better than older plant parts for protoplast isolation. Nagata and Takebe (1971) were of the view that in general, dark green leaf or a yellowish senescing leaf yield unstable protoplasts which degenerate during enzyme treatment.

Jia (1982) opined that protoplast from older leaves have low division frequencies or not divide at all. In contrary to that, Kaerlas *et al.* (1992) reported no differences in tolerance of young and old material to protoplasting and electroporation. They showed that survival rate of mesophyll protoplasts from older leaves was higher than that from younger leaves.

Young *in vitro* grown plants (Bajaj, 1972), young tissue and explants such as root tip (Xu *et al.*, 1982), hypocotyl (Glimelius, 1984), shoots (Russel and

McCown, 1986) and cotyledons (Hammat *et al.*, 1987) required low concentration of enzymes and a relatively short period of treatment as compared to large leaves from old or mature plants.

2.2.1.3 Pre-treatments of the donor tissue

The penetration of enzymes into leaves from green house is increased by pre-treating the lower epidermis with a soft nylon brush until the colour turned to light green (Shepard and Totten, 1977; Shahin, 1984).

Slicing of the plant material, particularly from *in vitro* cultures is the most common preparation before the enzyme incubation (Binding *et al.*, 1978). By a screening experiment, Foulger and Jones (1986) reconfirmed that slicing is the most efficient procedure with respect to the number of isolated protoplasts.

2.2.2 Osmoticum

Protoplasts released directly into standard cell culture medium will burst (Evans and Bravo, 1983). Hence, the pressure that is mechanically supported by the plant cell wall must be replaced with an osmoticum with appropriate osmotic pressure. The osmotic pressure between cell interior and exterior must be balanced or transfer of cells to a plasmolyzing solution will induce stress on the plant cell.

Osmotic pressure is manipulated by adding various sugars or sugar alcohols to the isolation and culture solution used for protoplasts. Most frequently, mannitol is used as the sole plasmolyzing agent. Sorbitol either alone or in combination with mannitol, glucose and sucrose has also been used successfully (Redenbaugh *et al.*, 1981 and David *et al.*, 1982).

Concentrations of plasmolyzing agents generally ranged from 0.4 to 0.7 M (Schenk and Hildebrandt, 1969; Kameya and Uchimiya, 1972). They were successful in satisfying the osmotic needs with salts such as KNO_3, KCl and $CaCl_2$.

Kao and Michayluk (1974) reported the use of 0.35 M sorbitol and mannitol to isolate protoplasts from suspension cultures of *Vicia hajastana, Pisum sativum, Glycine max* and *Hordeum vulgare*. David and David (1979) used 0.6 to 0.7 M glucose to isolate protoplasts from cotyledons of *Pinus pinaster*. Hurwitz and Agrios (1984) reported isolation of protoplasts from mature leaves of *Salix* spp. by using 11 per cent mannitol. Butt (1985) isolated protoplasts from juvenile leaves of *Alnus glutinosa* by using 11 per cent mannitol.

Ochatt (1993) reported the use of 13 per cent mannitol to isolate protoplasts from leaves, stems and roots of axenic plants of the hybrid ornamental shrub weigela x florida cv. Bristol Ruby. Xiao and Hirsch (1996) reported the use of 0.4 M mannitol for the effective isolation of protoplasts in the genus *Actinidia* Lindl. Karim and Adachi (1997) reported the use of 0.5 M mannitol for isolating protoplasts from mesophyll cells of *Allium cepa*. Philip *et al.* (1998) suggested 10 per cent sorbitol and 8 per cent sorbitol to isolate protoplasts from *in vitro* leaves of *Piper nigrum* and *P. colubrinum* respectively. Bang *et al.* (1999) reported isolation of protoplasts from *Bupleurum falcatum* suspension cultures by using 9 per cent mannitol.

The vitality of the protoplasts was increased by the addition of 5 to 10 mM Ca^{2+} ions as $CaCl_2$ or $Ca(NO_3)_2$ (Binding *et al.*, 1978, Fish and Karp, 1986). David *et al.* (1984) and Rao and Akins (1985) reported that calcium chloride in the range of 2 to 10 mM helped to maintain protoplast integrity. They further reported that inclusion of buffer component 2-[N-Morpholino] Ethane Sulphonic acid (MES) was beneficial for the isolation of protoplasts of forest trees.

Kaerlas *et al.* (1992) used 0.05 M $CaCl_2$ in the isolation of pea seedling protoplasts. Kik and Zaal (1993) reported use of 6.0 mM $CaCl_2$ $2H_2O$ to isolate protoplasts from mesophyll cells of *Brassica oleraceae*. Panis *et al.* (1993) reported the use of 7.0 mM $CaCl_2$. $2H_2O$ and 3 mM MES for isolating protoplasts from suspension cultures of banana. Peeters *et al.* (1994) reported isolation of

protoplasts from suspensions of cotton by using 0.147 per cent $CaCl_2$. Schnabl *et al.* (1999) isolated protoplasts from sunflower hypocotyl and broad bean guard cells by using 13.6 mM $CaCl_2$ and 3.59 mM MES.

2.2.3 Concentration of enzymes

Uchimiya and Murashige (1974) reported isolation of protoplasts from suspension cultures of tobacco cells by treating with one per cent cellulase 'Onozuka' and 0.2 per cent macerozyme. Ochatt (1993) reported isolation of protoplasts from leaves and stems of axenic plants of the hybrid ornamental shrub weigela x florida cv. Bristol Ruby by treating with 0.1 per cent pectolyase Y-23, one per cent cellulase Onozuka R-10 and one per cent hemicellulase. Protoplasts were isolated from root tissues by treating with two per cent meicelase, 0.03 per cent macerozyme R-10 and two per cent driselase.

Hansen and Earle (1994) isolated protoplasts from leaves of *Brassica oleraceae* by using two per cent cellulysin, 0.5 per cent driselase and one per cent macerozyme. Karim and Adachi (1997) reported the isolation of protoplasts from mesophyll cells of *Allium cepa* by using two per cent cellulase 'Onozuka' R-5 and 0.05 per cent pectolyase Y-23. Tamura *et al.* (1998) isolated protoplasts from calli of *Diospyros glandulosa* by using 0.5 per cent cellulase R-5 and 0.2 per cent macerozyme and from *D. kaki* by using 0.5 per cent cellulase R-5 and 0.05 per cent macerozyme R-10. Bang *et al.* (1999) reported the isolation of protoplasts from suspension cultures of *Bupleurum falcatum* by treating with cellulase two per cent and macerozyme 0.5 per cent. Protoplasts were isolated from nucellar callus of mango cv. Amrapali using 1.2 per cent cellulase, one per cent hemicellulase and 0.6 per cent pectinase (Ara *et al.*, 2000).

2.2.4 Time of incubation

Time required for the release of protoplasts differs with species, nature of starting material and the enzymes employed (Kirby *et al.*, 1989). Dilute enzyme

solutions needed longer incubation periods compared to more concentrated enzyme solutions (Smith and McCown, 1982).

The period of incubation of cells with enzyme has been varied considerably among investigators. Reusink and Thimamn (1965) used a period of one to two hour to isolate protoplasts from *Avena sativa* coleoptiles, whereas 10 to 12 hours incubation time was required to isolate protoplasts from *Daucus carota* callus (Grambow *et al.*, 1972).

Overnight incubation (12 h) is required for isolation of protoplast from *Alnus glutinosa* leaves (Huhtinen *et al.*, 1982), whereas 20 to 22 h incubation period was required to isolate protoplasts from *Fagus sylvatica* leaves (Ahuja, 1984). Butt (1985) reported an overnight incubation period for isolating protoplasts from *Salix* spp., whereas Kaerlas *et al.* (1992) reported an overnight incubation period for isolating protoplasts from pea seedlings.

Perales and Schieder (1993) isolated protoplasts from young leaves of apple by incubating in an enzyme mixture for 17 hours. Panis *et al.* (1993) reported that relatively long enzyme incubation periods (24 h) were needed for high protoplast densities (10^6 protoplasts ml^{-1}) from cell suspension cultures of *Musa* as compared with the four hours used in most isolation procedures.

The incubation period of 18 to 20 h was required to isolate protoplasts from cotyledons of *Brassica campestris* (Zhao *et al.*, 1994). Rybczynski (1997) isolated protoplasts from leaves of *Trifolium fragiferum* by incubating in an enzyme mixture for eight to ten hours.

Tican and Menczel (1998) reported an incubation period of 20 h for isolating protoplasts from cell suspensions of *Nicotiana africana*. Similarly, Schnabl *et al.* (1999) reported an overnight incubation period for isolating protoplasts from sunflower hypocotyl and broad bean guard cells.

2.2.5 pH

An optimum pH is essential for the effective isolation of protoplasts (Power and Cocking, 1970). pH of 5.6 has been suggested in the isolation of protoplasts in apple (Perales and Schieder, 1993), *Actinidia* spp. (Xiao and Hirsch, 1996) and *Moricanda nitens* (Tian and Meng, 1999).

Optimum pH of 5.8 has been reported for the effective isolation of protoplasts in *Glycine* (Schwenk *et al.*, 1981), *Musa* spp. (Panis *et al.*, 1993), *Allium cepa* (Karim and Adachi, 1997) and *Bupleurum falcatum* (Bang *et al.*, 1999).

2.2.6 Temperature

Temperature of 25°C has been suggested for the isolation of protoplasts from *Carica papaya* (Liu and Yang, 1983), *Theobroma cacao* (Thompson *et al.*, 1987), *Brassica oleraceae* (Kik and Zaal, 1993). *Nicotiana africana* (Tican and Menczel, 1998), *Helianthus annus* hypocotyl and *Vicia faba* guard cells (Schnabl *et al.*, 1999).

Philip *et al.* (1998) reported isolation of protoplasts at 21 ± 3°C in dark from leaves of *Piper nigrum* and *P. colubrinum*.

2.3 Purification of protoplasts

The purification of protoplasts is most frequently accomplished by repeated centrifugation and resuspension, usually in culture medium (David and David, 1979, Kirby and Cheng, 1979). For mesophyll protoplasts, that are extremely delicate, filtration results in excess cell breakage. Consequently, for a number of species, floatation has been used to purify mesophyll protoplasts (Gamborg *et al.*, 1981).

The enzyme treatment results in a mixture of undigested cells, components of broken or burst cells and protoplasts (Evans and Bravo, 1983). The

mixture should be partially purified to eliminate broken and undigested cells. They further reported that most frequently used purification technique is filtration centrifugation.

Tree protoplasts, particularly those isolated from conifer cell suspensions are extremely fragile (Kirby *et al.*, 1989). Centrifugation procedure frequently disrupt the fragile protoplasts. Simple gravity sedimentation under proper osmotic condition has been reported successful in purification of protoplasts from cell suspensions of loblolly pine (Teasdale and Rugini, 1983a and 1983b). They reported purification of protoplasts of loblolly pine (*Pinus taeda*) seedlings by simple washing which was achieved without damaging centrifugation.

Renfroe *et al.* (1986) suggested purification of protoplasts in cotton by floating protoplasts over sucrose solution (20.0 per cent w/v). However, Burrus *et al.* (1988) reported the purification of sunflower (*Helianthus annus* L.) protoplasts by floatation in 10 per cent ficoll gradient.

Batra and Dhingra (1990) purified *Eruca sativa* (an oil seed plant) protoplasts by low speed centrifugation (100 g*) in 20 per cent sucrose and repeated washing in osmoticum.

Kaerlas *et al.* (1992) reported purification of pea protoplasts isolated from germinating seedlings by passing through 53μm nylon mesh sieve, floating the protoplasts on the top of 1:1, 0.4 M sucrose : percoll cushion and finally washed by sedimenting twice in W_5 medium.

Yang *et al.* (1993) purified protoplasts from suspension cultures of durum wheat (*Triticum durum* Desf. cv. D 6962) by filtration through 53 μm and 38μm stainless steel meshes followed by washing thrice with a solution containing 20 mM $CaCl_2$ and 0.6 M mannitol.

Wang and Lorz (1994) reported purification of protoplasts from three-day-old suspensions of wild barley (*Hordeum murinum* L.) by filtering through 50 and 30 μm diameter sieves and pelleting the protoplasts by centrifugation at 800 rpm for seven minutes.

Xiao and Hirsch (1996) reported purification of mesophyll protoplasts of *Actinidia* spp. by passing through 78 μm nylon sieve, followed by centrifugation at 120 g* for five minutes and then layered on a CPW-24S (CPW salts with 0.7 M sucrose) bed. Centrifugation was done for five minutes at 100 g* and clean protoplasts recovered from the interphase were washed two times by centrifugation at 100 to 120 g* for five minutes, first with CPW-Na and then in culture medium without growth regulators.

Rybczynski (1997) reported purification of mesophyll protoplasts of *Trifolium fragiferum* by sieving through 45 μm nylon mesh filter. After two washings in CPW 13 M and centrifugation at 90 g* min^{-1}, protoplasts were washed by centrifugation in a 1:1 mixture of CPW13 M and KMP8 medium. For the last washing, only KMP8 culture medium was used.

Philip *et al.* (1998) reported purification of protoplasts by passing through a stainless steel mesh of 100 μm pore size followed by centrifugation at 750 rpm for *Piper nigrum* and 500 rpm for *P. colubrinum*.

2.4 Protoplast culture

2.4.1 Medium composition

The cell culture media most commonly used as a basis for protoplast culture are MS medium (Nagata and Takebe, 1971) and B_5 medium (Kao and Michayluk, 1975). The composition of basal media commonly used for perennial crop protoplast culture is given in Appendix I.

The detailed descriptions of the components of protoplast culture media have been reviewed by Eriksson (1977) and Gamborg (1977). The nutritional

requirements of cultured plant cells and protoplasts are very similar and so protoplast culture media are usually modifications of frequently used cell culture media.

Michayluk and Kao (1975) reported sucrose as the preferred carbon source for bromegrass protoplasts. Uchimiya and Murashige (1976) stated that tobacco protoplasts grow equally well on sucrose, cellobiose and glucose. Gamborg (1977) suggested glucose as the most preferred carbon source for most protoplasts. Zapata *et al.* (1981) suggested a mixture of sucrose and glucose in the ratio of 2:1 as the preferred carbon source for tomato.

Kao and Michayluk (1974) stated that in some cases preferred carbon source could also be the preferred osmoticum. David and David (1979) used MS media with 4.0 mM $CaCl_2$. $2H_2O$ for the culture of protoplasts from cotyledons of *Pinus pinaster.*

Huhtinen *et al.* (1982) used N_6 macro elements and MS micro elements as basal media for culturing *Alnus glutinosa* and *Alnus incanus* protoplasts. Ochatt *et al.* (1987) cultured protoplasts of colt cherry (*Prunus avium* x *Pseudoceracus*) in MS medium with 1.0 mg l^{-1} NAA, 0.25 mg l^{-1} BA and 0.5 mg l^{-1} Zeatin with agarose solidification.

Park and Son (1992) reported the culture of protoplasts in hybrid poplar (*Populus nigra* x *Populus maximowiczii*) by using MS medium lacking NH_4NO_3. MS basal medium supplemented with 2.2 μM BA, 2.6 μM NAA and 2.2 μM 2,4-D was used for protoplast culture of apple by Perales and Schieder (1993).

Karim and Adachi (1997) reported the use of BDS liquid medium (pH 5.8) containing three per cent sucrose, 0.5 M glucose, 5.0 mM $CaCl_2$, 250 mg l^{-1} casein enzymatic hydrolysate, 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA for the culture of protoplasts of *Allium cepa*.

Bang *et al.* (1999) cultured *Bupleurum falcatum* protoplasts on MS medium containing three per cent sucrose, nine per cent mannitol, 9.0 µM 2,4-D, 4.4 µM BA and 2.3 µM Kinetin at pH 5.8.

Saski *et al.* (1982) reported that reduction of sucrose concentration in the culture medium of *Cichorium intybus* dramatically enhanced the viability of the micro colonies. Chanabe *et al.* (1989) remarked that a reduction in sucrose concentration from two to 0.5 per cent allowed a two-fold increase in the colony yield of sunflower.

2.4.2 Modifications of the culture medium

In protoplast culture, it has been said that the concentration of ammonium salt in the MS medium is too high for the protoplast to survive (Kao *et al.*, 1973; Meyer, 1974; Uchimiya and Murashige, 1976).

Media have been devised for many species such as potato (Upadhya, 1975), tobacco (Caboche, 1980), tomato (Zapata *et al.*, 1981), rice (Toriyama and Hinata, 1985; Yamada *et al.*, 1986; Sun *et al.*, 1990), Chineese cabbage (Yamshigi *et al.*, 1988), *Piper nigrum* and *P. colubrinum* (Philip *et al.*, 1998) that are devoid of ammonium.

Yabe *et al.* (1989) tried modified MS medium (diluted NH_4NO_3 concentration) to clarify the effects of ammonium salt and MS salts on protoplast division. It showed that one-fourth dilution of MS salts with 200 mg l^{-1} NH_4NO_3 equivalent to 1/33 NH_4NO_3 concentration (50 mg l^{-1}) of the original MS medium (1650 mg l^{-1} NH_4NO_3) was most preferable.

Yin *et al.* (1993) opined that NH_4^+ was necessary for division and callus formation by *indica* rice protoplasts. Eriksson (1977) suggested that calcium concentration should be increased two to four times over the concentration

normally used for cell cultures as increased calcium concentration may be important for membrance stability.

Usually, pH used in protoplast culture is in the range of 5.6 to 5.8. Higher pH values have been reported to significantly enhance cell division in asparagus (Mackenzie *et al.*, 1973), pea (Gamborg *et al.*, 1975) and cowpea (Bharat and Rashid, 1980) protoplast derived cells. The survival rates of grape vine protoplasts were higher at initial pH of 5.4 to 7.0 compared to lower pH (Katsirdakis and Angelakis, 1992).

2.4.3 Plating density

High cell densities are beneficial for the initiation of mitosis. In potato, the applied densities ranged between 1.0×10^4 (Thomas, 1981) and 5.0×10^5 (Devries and Bokelmann, 1986).

Canas *et al.* (1987) reported an optimal plating density of 3.0×10^4 protoplasts ml^{-1} for olive (*Olea europaea* L.). The plating densities of protoplasts of tree species varied considerably from 5.0×10^3 protoplasts ml^{-1} in poplar and aspen (Russell and McCown, 1988) to 2.0×10^6 ml^{-1} in maritime pine (David and David, 1979).

Ochatt (1990) obtained best results for sour cherry (*Prunus cerasus*) when 0.25×10^6 protplasts ml^{-1} was plated. Tao *et al.* (1991) reported the best plating density of 0.1×10^6 protoplasts ml^{-1} for persimmon (*Diospyros kaki*).

Perales and Schieder (1993) suggested 1.0×10^5 protplasts ml^{-1} for apple as the most efficient plating density. Wang and Lorz (1994) opined that a plating density of 1.0×10^6 protoplasts ml^{-1} was optimal in wild barley (*Hordeum murinum* L.).

Anthony *et al.* (1995) reported the best plating density of 4.0×10^5 protoplasts ml^{-1} for cassava (*Manihot esculenta*). Xiao and Hirsch (1996)

suggested the best plating density of 2.0 x 10^5 protoplasts ml^{-1} for the genus *Actinidia* Lindl.

Rybczynski (1997) reported 1.0 x 10^6 protoplasts ml^{-1} for *Trifolium fragiferum* as the most efficient plating density. Tican and Menczel (1998) suggested the best plating density of 4.0 x 10^4 protoplasts ml^{-1} for *Nicotiana africana*. Similarly, Morgan (1999) reported 2.0 x 10^5 protoplasts ml^{-1} for *Cyclamen persicum*.

2.4.4 Plating technique

2.4.4.1 Liquid culture

Most frequent procedure employed with tree protoplasts is liquid culture (David *et al.*, 1982). Liquid culture was developed by Kao *et al.* (1971).

Liu and Yang (1983) reported liquid culture as best method for papaya leaf derived protoplasts. High plating efficiency in white spruce (*Picea glauca*) has been reported using a modification of the agarose bead procedure (Shillito *et al.*, 1983). In this procedure, protoplasts are suspended in a culture medium at a desirable density and then cultured as small droplets or as cell suspension, either with or without moderate agitation (Teasdale and Rugini, 1983a and b).

Vasil and Vasil (1987) reported that it was difficult to determine plating efficiencies accurately in liquid cultures. Panis *et al.* (1993) stated that liquid cultures were not suitable for *Musa* protoplasts since they tended to aggregate, which made the observations difficult. When cultured in liquid medium, apple protoplasts divided initially but soon the dividing cells started to aggregate resulting in only a few protocalli (Perales and Schieder, 1993).

In liquid cultures of *Allium cepa*, protoplast aggregation occurred to varying degree (Karim and Adachi, 1997). They suggested that, this problem could

be obviated by embedding the protoplasts in two per cent agarose beads. Philip *et al.* (1998) reported liquid culture for *Piper nigrum* and *P. colubrinum*.

2.4.4.2 Agar culture

Freshly isolated protoplasts were usually embedded into culture media which were jelled by agar (Shepard and Totten, 1977, Carlberg *et al.*, 1983, Haberlach *et al.*, 1985) or by agarose (Shepard, 1980, Binding *et al.*, 1986, 1987 and 1988) or by alginate (Rentschler *et al.*, 1987).

Agar culture has been reported in various crops like apple (Perales and Schieder, 1993), durum wheat (Yang *et al.*, 1993), banana (Panis *et al.*, 1993), *Hordeum murinum* L. (Wang and Lorz, 1994), *Actinidia* spp. (Xiao and Hirsch, 1996), *Allium cepa* (Karim and Adachi, 1997), *Cyclamen persicum* (Morgan, 1999) etc.

In some cases, regeneration was initiated in liquid culture media and the plastocytes and cell clusters were embedded into media containing final agar concentrations of 0.1 to 0.2 per cent agar after 2 to 14 days (Binding *et al.*, 1978, Thomas, 1981, Bokelmann and Rosset, 1983, Devries and Bokelmann, 1986, Debnath *et al.*, 1986, Kaerlas *et al.*, 1992).

2.4.5 Culture conditions

2.4.5.1 Illumination and culture temperature

Illumination and temperature affects the culture of protoplasts (Li and Chen, 1990). Culture room temperature of 24-28°C is generally suitable except for temperature sensitive species. Banks and Evans (1976) opined that generally high light intensity inhibited protoplast growth when applied from the beginning of the culture.

Temperature of 25±1°C and darkness has been suggested for the protoplast culture of *Beta vulgaris* (Hall *et al.*, 1993), *Triticum durum* (Yang

et al., 1993), Hordeum murinum (Wang and Lorz, 1994), *Brassica campestris* (Zhao *et al.*, 1994), *Actinidia arguta* var. arguta, *A. arguta* var. purpurea, *A. arguta* cv. Issai, *A. deliciosa*, *A. kolomikta* and *A. polygama* (Xiao and Hirsch, 1996), *Allium cepa* (Karim and Adachi, 1997) and *Bupleurum falcatum* (Bang *et al.*, 1999).

2.4.6 Dilution of the protoplast culture medium

Chupeau (1989) remarked that osmotic potential of protoplast isolation and culture media has to be at the lowest value. As medium evaporates, osmotic potential has to be reduced gradually, thus enhancing the actual concentration of various components of the culture medium and especially that of the osmotic stabilizers.

Shillito *et al.* (1983) reported that frequent replacement of the surrounding medium reduced inhibitory and toxic substances released by the developing cells. It was showed that reduction of the osmoticum increased the growth rate of cells and decreased the amount of phenolic compounds released by the growing calli.

Reduction of the osmoticum by dilution of the culture medium has been reported in various crops like *Brassica nigra* (Narasimhulu *et al.*, 1993), hybrid ornamental shrub, weigela x florida cv. Bristol Ruby (Ochatt, 1993), *Medicago polymorpha* (Scarpa *et al.*, 1993), *Oryza sativa* (Yin *et al.*, 1993), *Brassica campestris* (Zhao *et al.*, 1994), *Actinidia* sp. (Xiao and Hirsch, 1996), *Trifolium fragiferum* (Rybczynski, 1997), *Moricanda nitens* (Tian and Meng, 1999) etc.

2.4.7 Cell wall formation

Rentschler (1977) stated that cell wall regeneration is a pre-requisite for nuclear and cell division. The studies by Giles (1972) revealed that rate and regularity of cell wall regeneration depend on the plant species and the state of

differentiation of donor cells used for protoplast isolation. He further reported that protoplasts from leaf mesophyll cells of *Nicotiana, Datura, Petunia* and *Brassica* formed new cell walls quickly. Within 24 hours, the spherical protoplasts became oval, as viewed on the light microscope and the cell wall could be detected by calcoflour white (CFW) stain. On the other hand, leaf protoplasts of cereals and legumes required about four days for cell wall regeneration. He observed that in some cases, mesophyll protoplasts were unable to form cell wall.

Smith and McCown (1982) reported that cell wall formation of *Betula* and *Rhododendron* as detected by asymmetrical shape assumed by viable protoplasts began within two to three days and was characteristic of all surviving cells after one week in culture.

Chen and Ku (1985) revealed that protoplasts from base tissue of the youngest leaf of banana did not proceed with cell wall regeneration and cell division and survived only for fifteen days.

Peeters *et al.* (1994) reported that protoplasts from two embryogenic cell lines of *Gossypium hirsutum* when cultured on feeder layers, first cell wall regeneration was observed two to three days after protoplast isolation as detected by calcoflour white.

Protoplasts of *Cyclamen persicum* elongated after four to five days in culture indicating cell wall formation and a few had undergone mitotic division (Morgan, 1999).

2.4.8 Protoplast division

Uchimiya and Murashige (1974) reported that 40 per cent of cultured tobacco protoplasts resumed cell division and produced cell clusters in eight days. Litz (1986) reported occassional division of protoplasts in papaya (*Carica*

papaya). Newell and Luu (1989) stated that upto 50 per cent of *Glycine* protoplasts divided within three to four days to give small cell colonies.

In *Linum usitatissimum*, first division occurred within 48 h in case of cotyledon, hypocotyls and cell suspension protoplasts and in 48 to 72 h in those from roots and shoots (Barakat and Cocking, 1989). Yasugi (1989) observed first division in orchid leaf protoplasts after five to seven days of culture.

Zhao *et al.* (1994) reported that cotyledonory protoplasts of *Brassica* sp. showed second division after six to seven days in culture and exhibited high frequency of cell division in seven-day-old cultures.

In *Bupleurum falcatum* protoplast culture, first cell division was observed after three days of culture, second division was subsequently observed after six days of culture and third division, fifteen days later (Bang *et al.*, 1999).

2.4.9 Callus formation and regeneration

Plant regeneration remains one of the principal technical obstacles for the use of cell fusion and hybridization for general application in plant breeding. If one of the protoplast species can be cultured to form dividing cells, the fusion product can also be expected to divide, assuming the occurrence of synchronous mitosis of the two nuclei (Gamborg and Dunn-Colemann, 1983 and Chakravarti and Scott, 1991).

Kumar *et al.* (1981) reported callus formation from mesophyll protoplast culture of *Brassica campestris* cv. Toria. Vardi *et al.* (1982) reported callus proliferation and plant regeneration from protoplasts of sour orange (*Citrus aurantium*), lemon (*C. limon*) and grape fruit (*C. paradisi*).

Sticklen *et al.* (1985a and b) reported the formation of callus and plantlet regeneration in *Ulmus* sp. Shoot bud regeneration from protoplasts of *Santalum album* was reported by Rao and Chadha (1986). Russel and McCown

(1988) reported the formation of callus and plantlets by protoplast culture in *Populus tremula*.

Chen and Chen (1992) reported the development of somatic embryos directly from protoplasts of crica after plating. Protoplast derived somatic embryos proliferated rapidly through direct somatic embryogenesis on modified MS medium with 1.0 mg l^{-1} ABA and developed into plantlets on transfer to medium without plant growth factors.

According to Wang *et al.* (1995) multiple shoots were produced from protoplast derived callus of simon poplar (*Populus simonii*) after culture on MS medium containing 4.44 µM BA, 2.3 µM kinetin, 2.8 µM zeatin and 0.54 µM NAA. The shoots of two to three cm height were isolated from the calli and rooted on half strength MS medium.

2.5 Protoplast fusion

Attempts to hybridize somatic cells were made early in the century by Winkler, Kuster and Michel (Power *et al.*, 1970, Melchers and Labib, 1974). Michel (1937) demonstrated the fusion of homospecific and heterospecific protoplasts after their treatment with sodium nitrate solution. However, fusion was rare and subsequent fusion products could not be cultured (Power *et al.*, 1970).

2.5.1 Fusion of protoplasts with Poly Ethylene Glycol (PEG)

Kao and Michayluk (1974) reported that aggregation and fusion of tree protoplasts could be induced by concentrated solutions of high molecular weight PEG (MW 1500 to 6000) in the presence of Ca^{2+} ions. This technique was reconfirmed by other researchers (Wallin *et al.*, 1974; Kartha *et al.*, 1974; Burgess and Fleming, 1974).

Kao *et al.* (1974) remarked that higher fusion frequencies were usually obtained, if the PEG was eluted with a high pH and high calcium ion solution at

pH 10.5 followed by washing the treated protoplasts with protoplast culture medium. After the treatment, they found up to 50 per cent heterokaryotes of soyabean (+) pea in the protoplast population.

Fusion by the action of polyethylene solution, Ca^{2+} and high pH was reported by Buttenko and Kuechko (1980), Binding *et al.* (1982), Barsby *et al.* (1984), Austin *et al.* (1985) and Debnath and Wenzel (1987). Binding *et al.* (1982) reported PEG as harmful to potato protoplasts.

A hypotonic solution with a proper osmotic strength at near neutral pH for eluting PEG and providing an osmotic shock was just as effective as a high pH Ca^{2+} ion solution to elute PEG to induce protoplast fusion (Kao and Saleem, 1986, Takahashi *et al.*, 1986).

Fujita and Tabata (1986) reported that protoplasts from cultured cells of *Lithospermum*, *Coptis* and *Duboisia* which were very difficult to fuse by PEG or electroshock could be fused by a combination of PEG and a hypotonic treatment.

Enomoto and Ohyama (1989) compared protoplast fusion between lettuce and Philadelphia fleabane (*Erigerson philadelphicus* L.) by using PEG and dextran methods. The former showed superior performance and a rate of 14 per cent fusion was obtained using 50 per cent concentration of PEG.

MATERIALS AND METHODS

MATERIALS AND METHODS

The study entitled "Parasexual hybridization of *Piper nigrum* and *Piper colubrinum* through protoplast fusion" was carried out in the Department of Plantation Crops and Spices and the Plant Tissue Culture laboratory of the Center for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara between September, 1998 to April, 2000. The procedures reported for the multiplication of black pepper *in vitro* using nodal segments (Joseph *et al.*, 1996) and callus mediated organogenesis in black pepper (Nazeem *et al*, 1990) and *P. colubrinum* (Babu *et al.*, 1996) were employed to produce axenic cultures of *P. nigrum* and *P. colubrinum*. From these axenic plants, leaves were used for protoplast studies. The materials used and the methodology adopted in this study are described below.

3.1 Culture medium

The MS medium suggested by Murashige and Skoog (1962) was used in the present investigations. Composition of the medium is furnished in Appendix II.

3.1.1 Preparation of the medium

All the chemicals of AR grade, used as ingredients in the MS medium were procured from M/s Merck India Ltd., Sisco Research Laboratories Pvt. Ltd., British Drug House and Sigma Ltd. Borossilicate glassware of Corning, Vensil and Borosil brands were used. The glassware were cleaned by soaking in a solution of potassium dichromate in sulphuric acid for half an hour. Later the glassware were washed with jets of tap water to remove all traces of potassium dichromate solution. Then, they were further cleaned by 0.1 per cent teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water. They were then dried in hot air oven for 24 hours and later stored in dust free place for further use.

The medium was prepared following the standard procedure adopted by Gamborg and Shyluk (1981). The stock solutions of major and minor elements were prepared and stored in pre-cleaned amber coloured bottles in refrigerated conditions. The stock solutions of growth regulators at 1000 mg l^{-1} were prepared and stored under refrigeration.

A cleaned steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. Little amount of distilled water was added to it and later required quantities of sucrose (carbohydrate source) and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted in between 5.5 to 5.8 using 0.1 N NaOH or HCl.

The medium was solidified with 0.75 per cent (w/v) good quality agar after adjustment of pH. The medium was stirred and heated to melt the agar and was poured when hot into small test tubes (15 x 2.5 cm) at the rate of 15 ml each and to bigger test tubes (200 mm x 38 mm) at the rate of 60 ml each. The test tubes were tightly plugged with non-absorbent cotton.

The media were sterilized in an autoclave by applying a pressure of 15 psi for 20 minutes (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in culture room till further use.

3.2 Collection and preparation of explants

3.2.1 Source of explants

The explant sources used for the study included rooted cuttings of *P. nigrum* cv. Panniyur-1 and *P. colubrinum*.

Runners were rooted after giving a dip in 1000 ppm solution of IBA for 45 seconds and maintained in pots in the glass house of College of Horticulture, Vellanikkara. In order to reduce contamination *in vitro*, prophylatic sprays were given to these plants with fungicides, namely Emisan 0.3 per cent and Bavistin 0.1 per cent at weekly interval.

3.2.2 Preparation of explants

Nodal segments of 10 to 15 mm size were prepared from shoots of Panniyur-1 and tender leaves were taken from plants of *P. colubrinum* maintained in the glass house. Explants were wiped with cotton impregnated in 70 per cent ethanol as pre-treatment.

3.2.3 Surface sterilization

The nodal explants of Panniyur-1 were surface sterilized under aseptic conditions maintained inside a laminar air-flow cabinet. They were dipped in 0.1 per cent Emisan for 10 minutes with intermittant shaking and washed throughly in sterile water. The nodes were then treated with 50 per cent ethanol (two minutes) and then washed in sterile water. Followed by this, explants were soaked in 0.1 per cent mercuric chloride ($HgCl_2$) for 12 minutes with intermittant shaking. Thereafter, they were rinsed thrice with sterile distilled water.

Tender leaf explants of *P. colubrinum* were dipped in 0.1 per cent $HgCl_2$ for six minutes with occassional shaking. Later on, they were thoroughly washed three times with sterile water.

Surface sterilized explants were spread on sterile filter papers to drain inside the laminar air-flow chamber. Explants free from adhering water were trimmed at edges to remove all drying-off tissues.

3.3 Inoculation procedure

Inoculation was carried out under strict aseptic conditions inside the laminar air flow chamber. The floor of the work bench, inside the chamber was wiped thoroughly, with 100 per cent ethanol to remove any traces of dust or adhering dirt. Sterilized forceps, petridishes, surgical blades, knives and blotting papers were kept inside the chamber and the whole chamber was sterilized with UV light for 30 minutes. Surface sterilized explants were inoculated into half strength MS medium fortified with BAP and IAA (1.0 ppm each).

3.4 Culture conditions

Cultures were incubated in a culture room provided with 3000 lux flourescent light for a period of 16 hours followed by eight hours of dark period daily. Temperature at 26±2°C and humidity between 60 and 80 per cent were maintained to satisfy the physical conditions inside the culture room.

3.5 Subculture

Subculturing was done under aseptic conditions, similar to those employed during inoculation of explants. Three weeks after incubation, the sprouted nodal segments of *P. nigrum* (Plate 1) and induced calli of *P. colubrinum* (Plate 2) were subcultured into the medium of same composition for further proliferation.

3.6 Isolation of protoplasts

3.6.1 Enzyme mixture

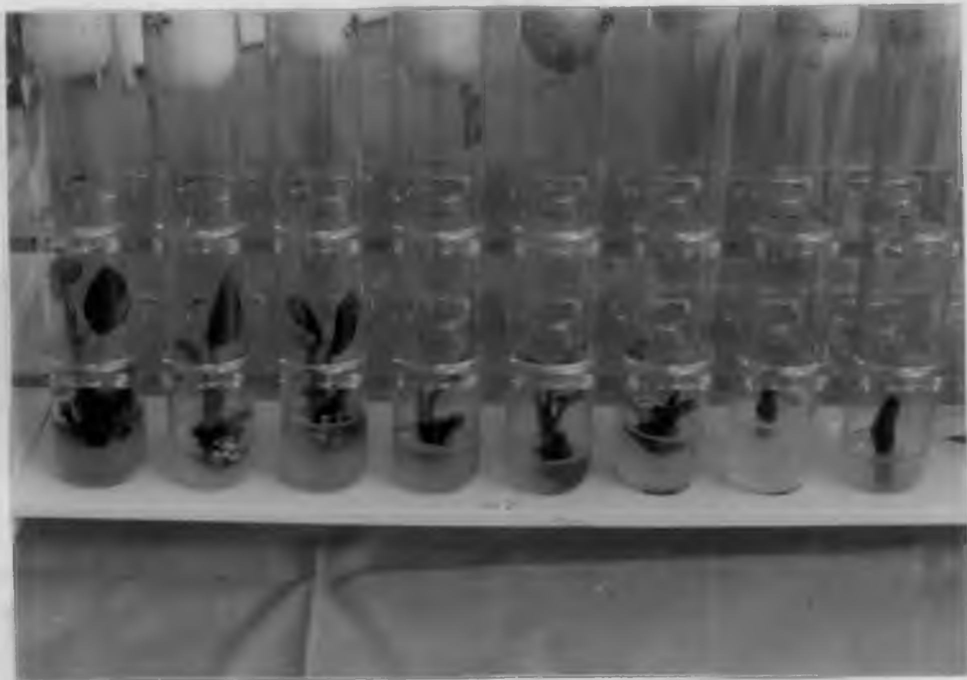
Enzymes, Cellulase from *Trichoderma viride* (M/s.Merck India Ltd.) and Pectinase from *Aspergillus niger* (M/s.Sisco Research Laboratory Pvt. Ltd.) were used at various concentrations and combinations for isolation of protoplasts from both the species (Table 1 and 2). All the experiments were replicated twice.

Table 1. Enzymes tried for protoplast isolation from *in vitro* leaves of *P. nigrum*

Treatment	Enzyme combination (%)
E_1	Cellulase 1.0 + Pectinase 0.280
E_2	Cellulase 0.9 + Pectinase 0.280
E_3	Cellulase 1.0 + Pectinase 0.155
E_4	Cellulase 1.0 + Pectinase 0.230
E_5	Cellulase 1.0 + Pectinase 0.500
E_6	Cellulase 1.1 + Pectinase 0.400
E_7	Cellulase 1.2 + Pectinase 0.310
E_8	Cellulase 1.4 + Pectinase 0.340

Plate 1. *In vitro* cultures of *Piper nigrum*

Plate 2. *In vitro* cultures of *Piper colubrinum*



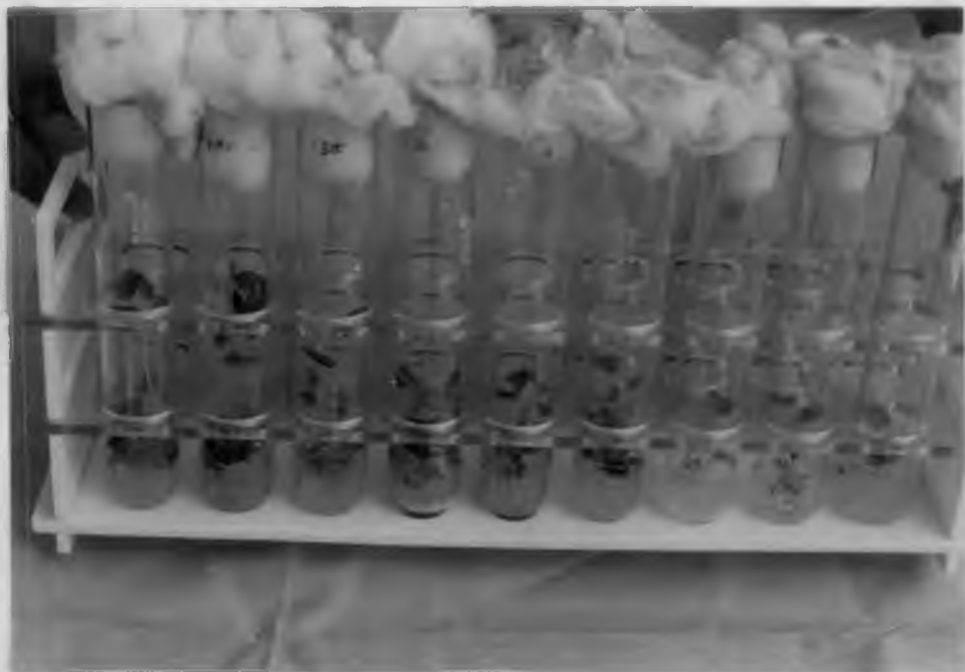


Table 2. Enzymes tried for protoplast isolation from *in vitro* leaves of *P. colubrinum*

Treatment	Enzyme combination (%)
T_1	Cellulase 1.0 + Pectinase 0.186
T_2	Cellulase 1.0 + Pectinase 0.217
T_3	Cellulase 0.9 + Pectinase 0.186
T_4	Cellulase 1.0 + Pectinase 0.155
T_5	Cellulase 1.0 + Pectinase 0.167
T_6	Cellulase 1.0 + Pectinase 0.500
T_7	Cellulase 1.1 + Pectinase 0.300
T_8	Cellulase 1.2 + Pectinase 0.240

3.6.2 Osmoticum

The osmotic stabilizers employed were mannitol and sorbitol at concentrations of 0.4, 0.5, 0.55, 0.6, 0.65 and 0.7 M respectively. Calcium chloride and MES buffer were also added at concentrations of 5.95 mM and 0.5 mM respectively. The levels of osmoticum tried for isolation of protoplasts are shown in Table 3. The corresponding protoplast yield was recorded in each enzyme concentration tried.

Table 3. Levels of osmoticum tried for protoplast isolation

Treatment	Levels of osmoticum (M)	Concentration of	
		Mannitol (M)	Sorbitol (M)
M_1	0.40	0.20	0.20
M_2	0.50	0.25	0.25
M_3	0.55	0.275	0.275
M_4	0.60	0.30	0.30
M_5	0.65	0.325	0.325
M_6	0.70	0.35	0.35

3.6.3 Preparation of isolation solution

The required quantity of enzymes were weighed and added to the osmotic solution and mixed thoroughly with constant stirring. The pH of the isolation solution was adjusted to 5.8 using 0.1N NaOH or HCl. Inside the laminar air flow cabinet, the isolation solution was filter sterilized (0.22 μm) and 10 ml each of the isolation solution was dispersed into sterile disposable petridishes (M/s.Tarsons) of 90 mm size.

3.6.4 Incubation

Green leaves were excised from the axenic cultures and transferred to sterile petridishes. Leaves were finely cut and transferred to isolation solution in the petridishes. The petridishes were sealed with parafilm and maintained in dark condition in the culture room. Using a Pasteur pipette, samples were drawn from the isolation solution under aseptic conditions, at hourly intervals and observed under the microscope for protoplast release.

3.7 Purification of protoplasts

The petridish was taken to laminar air flow chamber and parafilm was removed. The contents were sieved through a sterile nylon cloth of mesh size 50 μm to remove the undigested plant materials. Then the filtrate was transferred to sterile centrifuge tubes and then centrifuged in high speed refrigerated centrifuge from M/s. Kubota at varying centrifugation speeds (500 to 1000 rpm) and for different durations ranging from three to five minutes. Pellets of protoplasts were formed at the bottom of the centrifuge tubes. The supernatant was decanted and the osmoticum was added to the tubes under aseptic condition. The tubes were then swirled, closed and again centrifuged. The supernatant was removed again under aseptic conditions. This process was repeated twice. Then the protoplast pellet was layered over sucrose solution taken in another sterile centrifuge tube by carefully adding through the sides. Different density gradients were tried with sucrose at 12, 15 and 20 per cent concentrations, in this floatation method of protoplast

purification. Control treatment was performed using osmotic solution. The tubes were then centrifuged at 600 rpm for three minutes. The protoplast layer at the bottom and at the inter phase was carefully removed using a Pasteur pipette and diluted with osmoticum and again centrifuged at 600 rpm for three minutes. The supernatant was then decanted. The protoplasts pelleted at the bottom of the centrifuge tubes were diluted in 1:10 ratio with the osmotic solution.

3.8 Protoplast viability

The drop of protoplast suspension taken previously on the slide was mixed with 10 μl Evan's blue solution (0.01%) and observed under a magnification of 40x through a light microscope. The blue stained protoplasts were dead. All the fields on the slide were screened and the number of total protoplasts and stained protoplasts were counted. The number of living (unstained) protoplasts was computed by substracting the number of stained protoplasts from the number of total protoplasts. It was then expressed as the percentage of total protoplasts counted. This was denoted as protoplast viability.

Number of living protoplasts = (Total number of protoplasts) - (Number of stained protoplasts)

3.9 Protoplast culture

Purified protoplasts were suspended in modified half strength MS medium (Philip *et al.*, 1998) under sterile conditions at densities ranging between 10^4 to 10^5 protoplasts ml^{-1} in sterile petridishes. Both liquid and solid media were tried. The composition of the medium is given in Appendix III. Mannitol and sucrose were used as the carbon source. BAP, IAA and 2,4-D (0.5 ppm each) were used as growth regulators. The petriplates were sealed with parafilm and incubated in dark at 25°C. The medium was renewed by adding fresh culture media at six days interval. The protoplasts were checked for their development at six days interval.

3.10 Protoplast fusion

Under sterile conditions of laminar flow, two drops (200 µl) containing about 1 x 10^5 protoplasts ml^{-1} of *P. nigrum* and *P. colubrinum* were placed in a glass slide inside a petridish. The protoplast suspension was allowed to settle for ten minutes at room temperature in order to form a thin layer. Four microdrops (each 50 µl) of PEG 6000 (Poly Ethylene Glycol), 10 g in 20 ml double distilled water (50 per cent) containing 0.7 mM KH_2PO_4, 10 mM $CaCl_2.2H_2O$ and 0.1 M glucose at pH 5.8 were added to the protoplast preparation. Protoplasts were incubated in PEG for 20, 30 and 40 minutes respectively. After that, three successive dilutions were performed at five minute intervals by adding 4000 µl of protoplast culture medium (PCM) to each drop of PEG-protoplast suspension. One more method was also tried in which PEG was eluted using high pH calcium ion solution containing 0.3 M glucose, 0.05 M $CaCl_2.2H_2O$ and 0.05 M glycine followed by washing the treated protoplasts with PCM. A drop of the protoplast suspension was taken in a glass slide and observed under the microscope.

RESULTS

RESULTS

The results of the study "Parasexual hybridization of *Piper nigrum* and *Piper colubrinum* through protoplast fusion are explicited in this chapter. Details of protoplast isolation, purification, culture and fusion studies of *P. nigrum* and *P. colubrinum* are described separately.

I. *Piper nigrum*

4.1 Osmoticum levels

4.1.1 Influence of osmotic potentials and duration of incubation on protoplast isolation with E_1

The effect of osmotic potentials and duration of incubation on protoplasts were studied by observing the intact protoplast yield and percentage of viable protoplasts.

4.1.1.1 Influence on intact protoplast yield

Out of the six levels of osmoticum viz. 0.4, 0.5, 0.55, 0.6, 0.65 and 0.7 M tried to compare the yield of protoplasts with E_1, highest yield of 6.5×10^4 protoplasts ml^{-1} was observed at 0.6 M after an incubation time of 19 hours (Table 4). The mean yield of protoplasts was highest at 0.6 M (4.03×10^4) followed by 2.2×10^4 protoplasts ml^{-1} at 0.65 M (Fig.1). Since, the release of protoplasts was better at 0.6 and 0.65 M in subsequent experiments osmolarity of the isolation solution was maintained at 0.6 and 0.65 M.

4.1.1.2 Influence on protoplast viability

Cent per cent viability of protoplasts was recorded after 21 hours of incubation at 0.55 and 0.65 M osmoticum (Table 5). Maximum mean viability was recorded at 0.55 M (99.45%) followed by 0.65 M (99.10%).

Table 4. Influence of varying duration of incubation and osmotic potential on intact protoplast yield (x 10^4) of *Piper nigrum* with E_1

E_1 / Osmoticum (M)	Duration of incubation (h)							Mean
	17	18	19	20	21	22	23	
0.40	0.74	1.20	1.56	2.04	2.42	3.02	2.63	1.94
0.50	0.85	1.41	2.06	3.70	1.50	2.35	2.64	2.07
0.55	1.01	1.64	1.73	1.84	1.80	2.70	2.20	1.84
0.60	1.21	2.64	6.50	4.02	3.90	4.65	5.35	4.03
0.65	0.92	2.38	2.42	2.52	2.58	2.50	2.10	2.20
0.70	0.86	1.42	2.60	1.50	1.48	1.43	1.51	1.54
Mean	0.93	1.78	2.81	2.60	2.28	2.78	2.73	2.27

Table 5. Influence of varying duration of incubation and osmotic potential on protoplast viability (%) of *Piper nigrum* with E_1

E_1 / Osmoticum (M)	Duration of incubation (h)							Mean
	17	18	19	20	21	22	23	
0.40	99.21	99.45	99.32	98.74	98.23	99.07	99.47	99.07
0.50	99.52	99.26	99.52	98.32	98.29	97.42	95.49	98.26
0.55	99.28	99.41	99.34	99.40	100.00	99.21	99.56	99.45
0.60	98.84	98.37	98.91	98.04	99.46	99.06	98.14	98.68
0.65	99.65	99.43	98.27	99.16	100.00	98.48	98.73	99.10
0.70	99.37	99.09	99.18	99.24	98.53	98.46	96.89	98.68
Mean	99.31	99.16	99.09	98.81	98.08	98.61	98.04	98.87

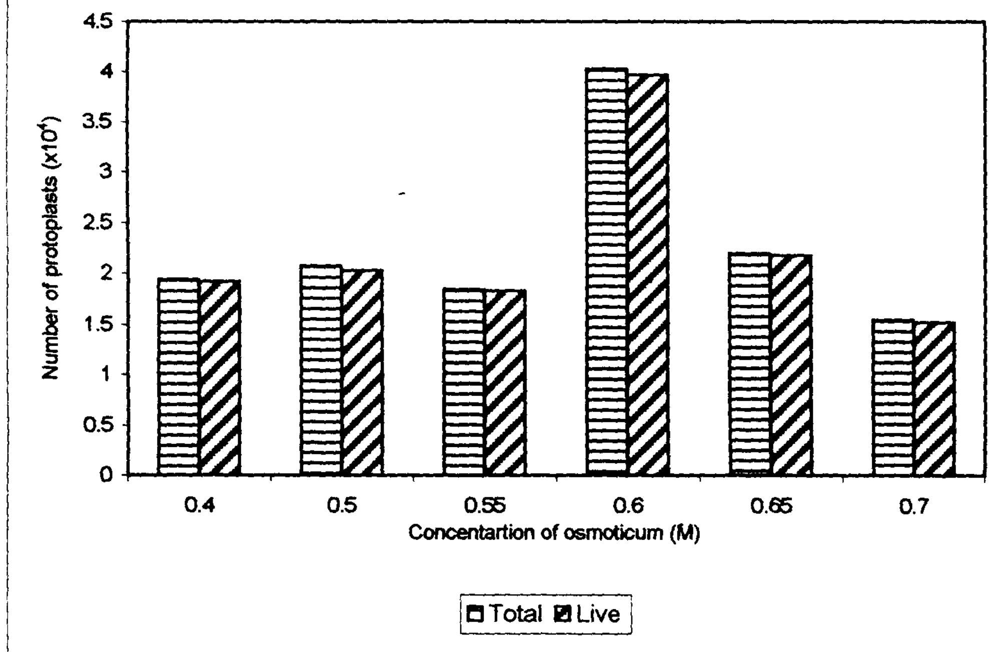


Fig. 1. Influence of concentration of osmoticum in enzyme solution (E1) on total and live protoplasts of Piper nigrum during 17 to 23h
Number of protoplasts (x10⁴)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0.4
0.5
0.55
0.6
0.65
0.7
Concentartion of osmoticum (M)
Total
Live

4.2 Enzymes treatments

4.2.1 Influence of enzymes and duration of incubation on yield of protoplasts at 0.6 M

Protoplast isolation was initiated during 17 h of incubation with E_1 (Plate 3), E_3 and E_4 (Table 6). At 15 h of digestion, isolation of protoplasts was observed in the descending order of magnitude in E_5, E_7, E_8 and E_6. The enzyme combination E_5 (2.16 x 10^4) also recorded higher yield at 17 h of incubation. Maximum protoplast yield at 19 h of incubation was registered with E_1 (6.5 x 10^4). During 21 h of incubation, the yield of protoplast was bestowed with the highest value in E_8 (6.9 x 10^4). This treatment exhibited the highest protoplast isolation efficiency among all the enzyme combinations tested at 0.6 M (Plate 4 and Fig.2). At 23 h of incubation, protoplast yield had shown a decreasing trend with increase in concentration of enzymes. Comparatively low protoplast yields were vested with E_3, E_4 and E_6 enzyme combinations.

4.2.2 Influence of enzymes and duration of incubation on viability of protoplasts at 0.6 M

During 15 h of incubation, the enzyme treatment E_8 (98.55%) was vested with increased protoplast viability (Table 7). While considering 17 h of digestion, the enzyme treatment E_8 registered with protoplast viability of 93.31 per cent. With regards to 19 h of incubation, the enzyme combination E_3 explicited 99.19 per cent protoplast viability. During 21 h of incubation, E_1 expressed protoplast viability of 99.46 per cent and this treatment recorded the highest viability among all the enzyme combinations. At 23 h of digestion, higher protoplast viability was observed in E_3 (99.24%).

4.2.3 Influence of enzymes and duration of incubation on yield of protoplasts at 0.65 M

The enzyme treatment E_8 was emulated with higher protoplast density at 15 h (2.47 x 10^4), 17 h (4.12 x 10^4) and 19 h (4.79 x 10^4) of incubation (Table 8).

Table 6. Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper nigrum* at 0.6 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
E_1	-	1.21	6.50	3.90	5.35	3.39
E_2	-	-	2.45	2.70	2.51	1.53
E_3	-	0.26	0.45	0.53	0.42	0.33
E_4	-	0.34	0.39	0.75	0.53	0.34
E_5	1.89	2.16	2.24	1.92	-	1.64
E_6	0.72	1.05	0.75	0.71	-	0.65
E_7	1.37	1.46	2.21	3.10	-	1.63
E_8	1.25	2.00	4.06	6.9	-	2.84
Mean	0.65	1.06	2.38	2.56	1.10	1.54

Table 7. Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper nigrum* at 0.6 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
E_1	-	98.84	98.91	99.46	98.14	79.07
E_2	-	-	98.41	98.05	97.85	58.86
E_3	-	99.15	99.19	99.34	99.24	79.38
E_4	-	98.24	98.17	98.41	98.22	78.61
E_5	92.35	91.17	91.24	91.04	-	73.16
E_6	90.31	90.17	91.45	90.71	-	72.53
E_7	90.15	90.85	90.20	90.63	-	72.37
E_8	98.85	99.31	98.18	96.38	-	78.54
Mean	46.45	83.46	95.71	95.50	49.18	74.06

Table 8. Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper nigrum* at 0.65 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
E_1	-	0.92	2.42	2.58	2.10	1.60
E_2	-	-	1.82	1.68	1.63	1.02
E_3	-	0.41	0.48	0.37	0.25	0.30
E_4	-	0.32	0.45	0.72	0.36	0.37
E_5	1.46	1.51	1.74	1.68	-	1.28
E_6	0.64	0.92	0.83	0.77	-	0.63
E_7	1.09	1.17	1.89	2.75	-	1.38
E_8	2.47	4.12	4.79	1.85	-	2.64
Mean	0.71	1.17	1.80	1.55	0.54	1.15

Fig. 2. Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper nigrum* at 0.6 M

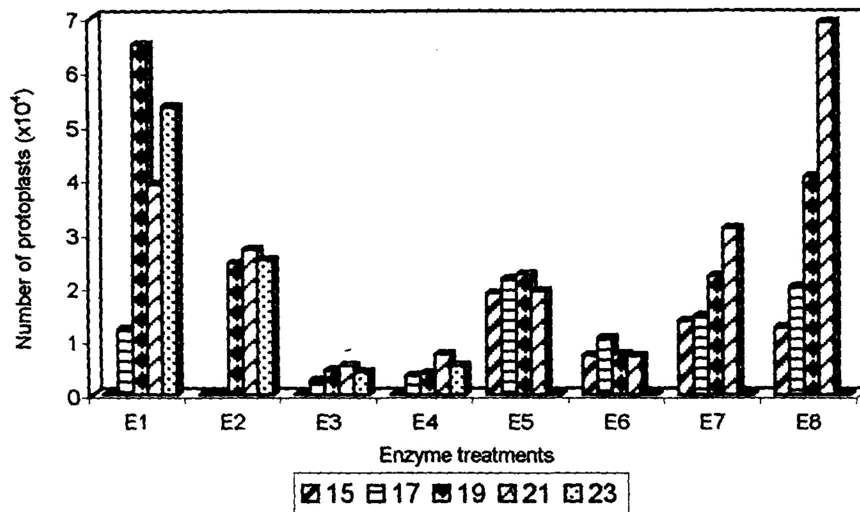


Fig. 3. Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper nigrum* at 0.65 M

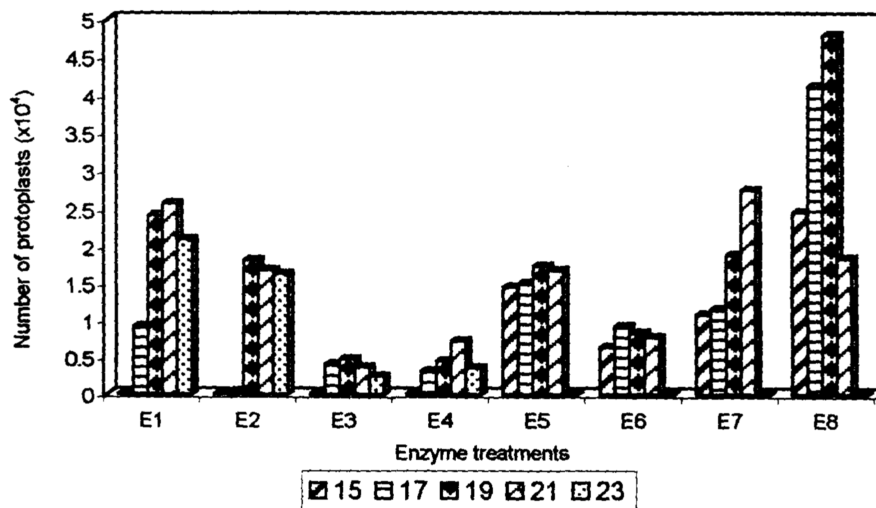
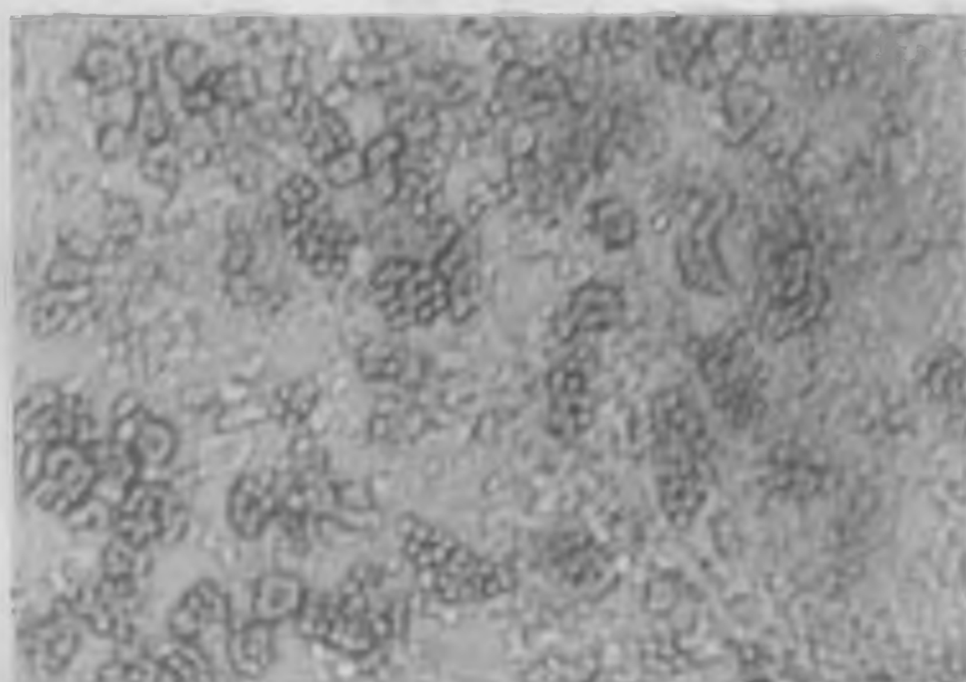
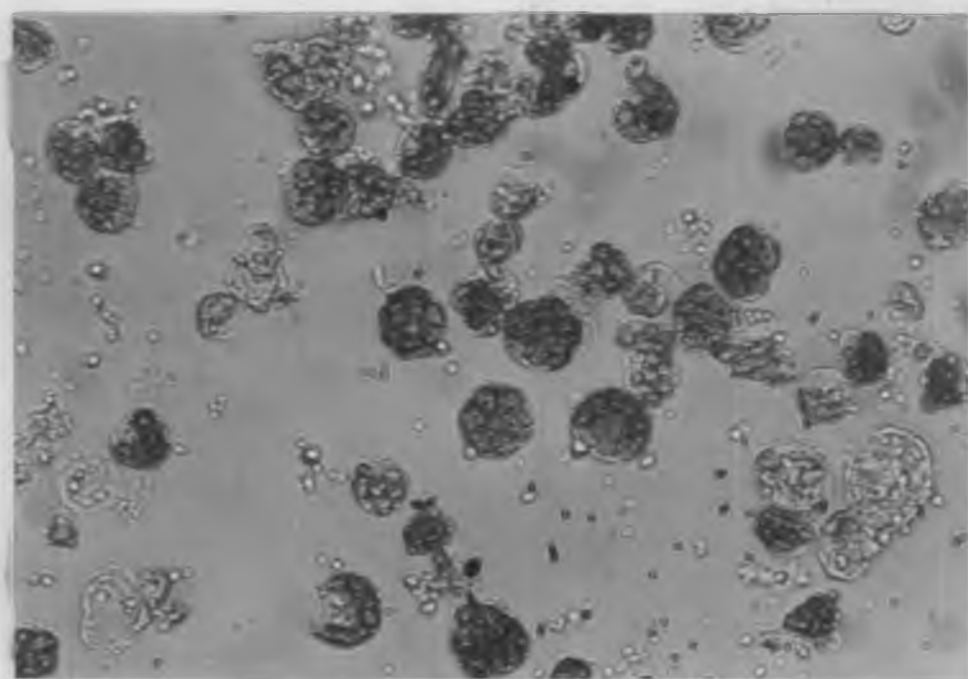


Plate 3. Interconnections between cells of *Piper nigrum* (E_1, 15 h, 0.6 M) (100 x)

Plate 4. Isolated protoplasts of *Piper nigrum* (E_8, 21 h, 0.6 M) (200 x)





This enzyme treatment at 19 h registered highest protoplast isolation efficiency among all the enzyme combinations tested at 0.65 M (Plate 5). In respect of 21 h of incubation, E_7 enzyme combination had shown 2.75 x 10^4 protoplast density. Whereas the maximum period of 23 h incubation yielded only 2.10 x 10^4 protoplasts at E_1.

4.2.4 Influence of enzymes and duration of incubation on viability of protoplasts at 0.65 M

Protoplast variability was found to be higher (97.42%) in E_8 as compared to other enzyme treatments at 15 h of digestion (Table 9). Enzyme combination E_3 noted with maximum viability at 17 h (99.71%) and 19h (99.24%) of enzymatic incubation. Among the treatments, E_1 recorded cent per cent viability at 21 h. At the incubation period of 23 h, highest protoplast viability was accounted with E_3 (99.17%).

4.3 Purification of protoplasts

4.3.1 Influence of different centrifugal speed and duration on protoplast yield

Highest yield of 4.5 x 10^4 protoplast ml^{-1} was noticed at a centrifugation speed of 1000 rpm for three minutes (Table 10). Protoplasts were not completely pelleted at lower centrifugation speeds as some of the protoplasts were still floating on the osmoticum.

4.3.2 Influence of density gradient on purification of protoplasts

Observations on the protoplast yield at different density gradients tested are given in Table 11. During centrifugation, protoplasts formed a band at the interphase between the sucrose solution and the osmoticum. In addition to this, some protoplasts pelleted at the bottom of the centrifuge tube.

Comparatively, high yields of protoplasts were obtained at the interphase of the two solutions compared to that in the pellet. Among the three

Table 9. Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper nigrum* at 0.65 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
E_1	-	99.65	98.27	100.00	98.73	79.33
E_2	-	-	98.30	97.75	98.06	58.82
E_3	-	99.71	99.24	99.10	99.17	79.44
E_4	-	98.09	98.12	97.51	97.15	78.17
E_5	91.46	90.53	91.81	90.15	-	72.79
E_6	90.48	90.68	90.14	90.05	-	72.27
E_7	90.74	90.02	90.48	90.31	-	72.31
E_8	97.42	97.12	98.71	98.07	-	78.26
Mean	46.26	83.22	95.63	95.36	49.13	73.92

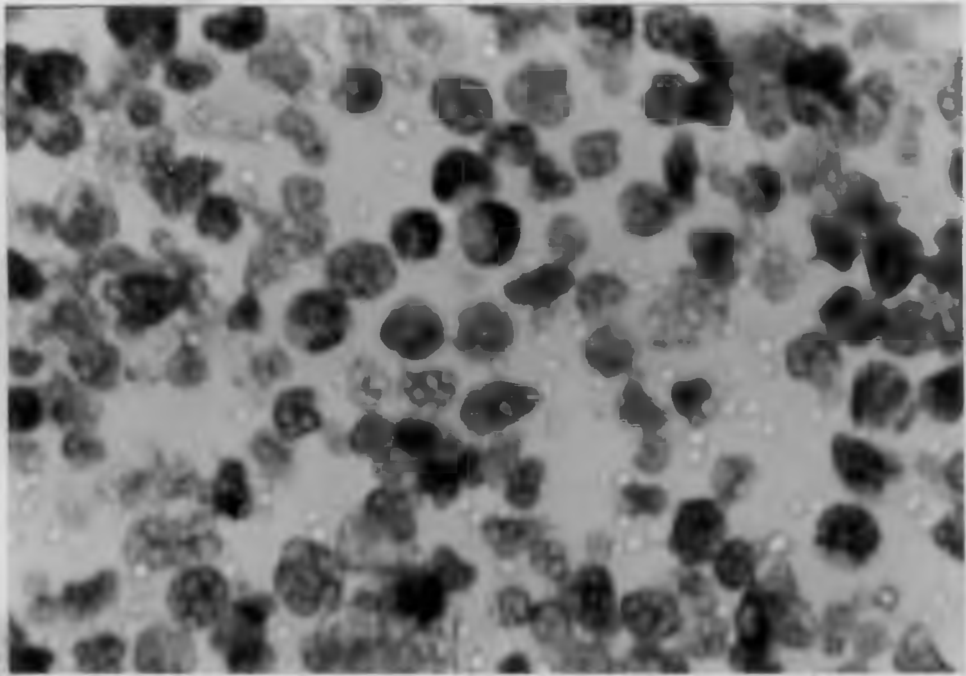
Table 10. Influence of different centrifugal speed and duration on protoplast yield ($x10^4$) of *Piper nigrum*

Duration	Speed (rpm)			Mean
	600	700	1000	
3	3.79	3.65	4.50	3.98
5	1.50	2.19	1.65	1.78
Mean	2.64	2.92	3.05	2.88

Table 11. Influence of density gradient on protoplast yield ($x10^4$) of *Piper nigrum*

Sucrose (%)	Protoplast yield ($x10^4$)	
	Interphase	Pellet
12	1.28	0.72
15	2.6	1.02
20	1.14	0.85
Control	-	3.10

Plate 5. Isolated protoplasts of *Piper nigrum* (E_8, 19 h, 0.65 M) (50 x)



density gradients tried, maximum yield (2.6 x 10^4) was observed at the interphase of 15 per cent sucrose concentration. The control treatment performed superior to the density gradient method with a protoplast yield of 3.1 x 10^4 protoplasts ml^{-1}

4.4 Protoplast size

There was no homogeneity in the size of protoplasts. Protoplast size varied between 8.46 µm to 23 µm.

II. *P. colubrinum*

4.5 Osmoticum levels

4.5.1 Influence of osmotic potential and duration of incubation on protoplast isolation with T_1.

The effect of osmotic potentials and duration of incubation on protoplasts were studied by observing the intact protoplast yield and percentage of viable protoplasts.

4.5.1.1 Influence on intact protoplast yield

Out of the six levels of osmoticum viz. 0.4, 0.5, 0.55, 0.6, 0.65 and 0.7 M tried to compare the yield of protoplasts (Table 12) highest yield (7.7 x 10^4) was obtained at 0.65 M osmotic potential after an incubation time of 20 hours. The mean yield of protoplasts was higher (4.61 x 10^4) at 0.6 M followed by 0.65 M (4.19 x 10^4) (Fig.4) and hence in further studies, osmoticum levels were maintained at 0.6 and 0.65 M.

4.5.1.2 Influence on protoplast viability

Cent per cent viability of protoplasts was noticed with 0.4 M osmoticum at 18 hours of incubation (Table 13). Mean values showed that maximum viability of 97.61 per cent was recorded with 0.6 M osmoticum.

Table 12. Influence of varying duration of incubation and osmotic potential on protoplast yield (x 10^4) of *Piper colubrinum* with T_1.

T_1 / Osmoticum (M)	Duration (h)							
	17	18	19	20	21	22	23	Mean
0.40	4.21	6.12	5.04	4.10	3.40	2.81	1.60	3.89
0.50	3.10	3.70	7.30	5.09	3.04	2.91	2.41	3.93
0.55	3.62	3.48	3.08	5.50	2.12	1.87	1.66	3.04
0.60	4.29	3.04	4.24	6.10	5.81	4.96	3.87	4.61
0.65	2.90	2.40	2.16	7.70	6.60	4.00	3.59	4.19
0.70	2.60	3.60	3.90	4.69	1.70	1.60	0.85	2.70
Mean	3.45	3.72	4.29	5.53	3.77	3.03	2.33	3.73

Table 13. Influence of varying duration of incubation and osmotic potential on protoplast viability (%) of *Piper colubrinum* with T_1.

T_1 / Osmoticum (M)	Duration (h)							
	17	18	19	20	21	22	23	Mean
0.40	98.71	100.00	97.23	97.51	97.72	96.14	95.47	97.54
0.50	97.84	97.47	97.21	97.52	97.51	97.43	97.21	97.45
0.55	98.41	96.35	96.14	97.15	96.42	96.86	96.56	96.84
0.60	98.28	98.18	97.12	97.25	97.35	97.74	97.36	97.61
0.65	98.19	97.25	97.54	97.52	97.78	96.67	96.15	97.30
0.70	98.23	98.15	99.12	97.24	98.48	96.15	95.28	97.52
Mean	98.27	97.90	97.39	97.36	97.54	96.83	96.33	97.37

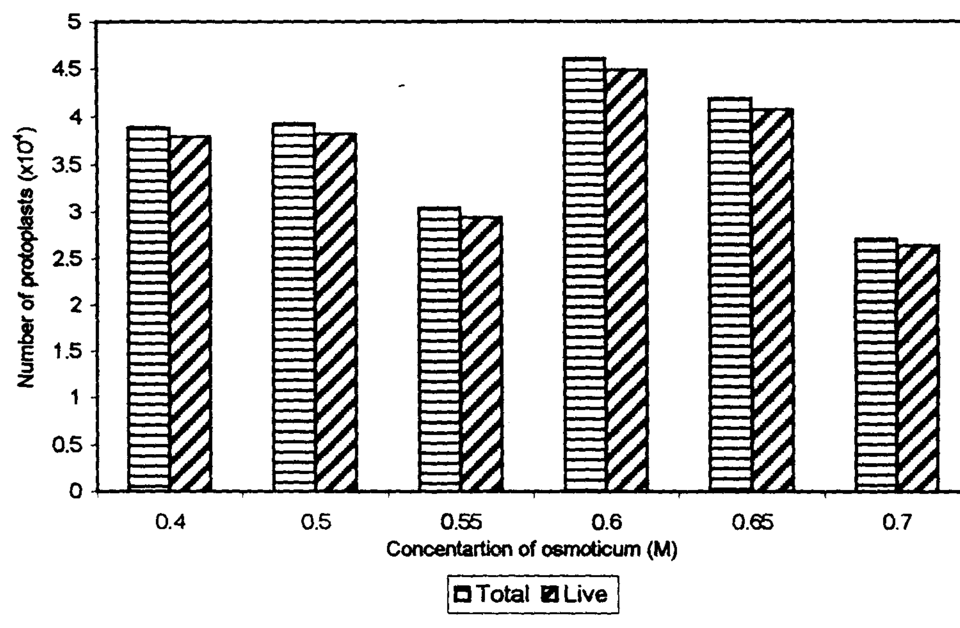


Fig. 4. Influence of concentration of osmoticum in enzyme solution (T_1) on total and live protoplasts of *Piper colubrinum* during 17 to 23h
Number of protoplasts ($x10^4$)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0.4
0.5
0.55
0.6
0.65
0.7
Concentartion of osmoticum (M)
Total
Live

4.6 Duration of incubation

4.6.1 Influence of duration of incubation on protoplast isolation with T_2

The effect of different duration of digestion on protoplasts were studied by observing the yield and viability of protoplasts at 0.6 and 0.65 M osmotic potentials.

4.6.1.1 Influence on protoplast yield

Protoplasts were found to be released at seven hours of incubation with T_2 (Table 14). Highest yield was observed during 21 h of incubation (13×10^4) at 0.6 M followed by an yield of 8.12×10^4 protoplasts ml^{-1} during 17 h of incubation at 0.65 M osmotic potential. However, when incubation was continued beyond 21 h, complete digestion of protoplasts was observed. As maximum protoplast yields were recorded during 17 to 21 h of incubation, further experiments were given the incubation periods between 15 to 23 h of digestion.

4.6.1.2 Influence on protoplast viability

Viability of protoplasts was found to be maximum after 15 h of incubation (94.74%) at 0.6 M followed by 94.26 per cent viability at 10 h of incubation at 0.65 M, osmoticum (Table 15).

4.7 Enzymes

4.7.1 Influence of enzymes and duration of incubation on yield of protoplasts at 0.6 M

Protoplast isolation was observed at 17 h of incubation in T_1 (Plate 6), T_3, T_4 and T_5 while it was initiated before 15 h with T_2, T_6, T_7 and T_8 (Table 16) enzyme combinations in *P. colubrinum*. Protoplast density was found to be high in T_2 (5.08×10^4) at 15 h of digestion. The enzyme combination T_8 had shown higher protoplast yield at 17 h (5.21×10^4) and 19 h (8.10×10^4). During 21 h, higher yield of protoplast was accounted with T_2 (13×10^4) (Plate 7 and Fig. 5). This

Table 14. Influence of varying duration of incubation on protoplast yield (x 10^4) of *Piper colubrinum* with T_2

T_2 / Osmoticum (M)	Duration (h)							Mean
	7	10	15	17	19	21	23	
0.60	2.50	3.74	5.08	5.12	6.30	13.00	-	5.11
0.65	2.89	4.21	6.37	8.12	7.27	7.04	-	5.12
Mean	2.70	3.98	5.73	6.62	6.79	10.02	-	5.12

Table 15. Influence of varying duration of incubation on protoplast viability (%) of *Piper colubrimum* with T_2

T_2 / Osmoticum (M)	Duration (h)							Mean
	7	10	15	17	19	21	23	
0.60	93.75	93.42	94.74	92.76	93.51	92.52	-	80.1
0.65	94.17	94.26	94.17	92.53	92.18	92.11	-	79.92
Mean	93.96	93.84	94.46	92.65	92.85	92.32	-	80.01

Table 16. Influence of enzymes and duration of incubation on protoplast yield (x 10^4) of *Piper colubrinum* at 0.6 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
T_1	-	4.29	4.24	5.81	3.87	3.64
T_2	5.08	5.12	6.30	13.00	-	5.90
T_3	-	3.82	4.34	7.85	5.54	4.31
T_4	-	3.35	4.00	2.42	-	1.95
T_5	-	3.12	3.60	3.52	-	2.05
T_6	3.60	3.54	-	-	-	1.43
T_7	1.48	1.75	2.61	2.37	-	1.64
T_8	4.01	5.21	8.10	9.45	-	5.35
Mean	1.77	3.78	4.14	5.55	1.18	3.28

Fig. 5. Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper colubrinum* at 0.6M

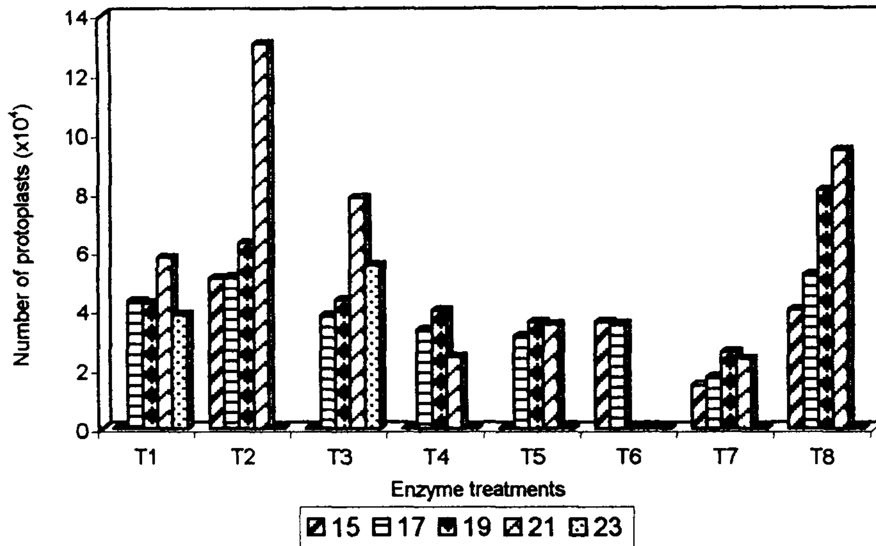


Fig. 6. Interaction of enzymes and duration of incubation on yield of protoplasts of *Piper colubrinum* at 0.65M

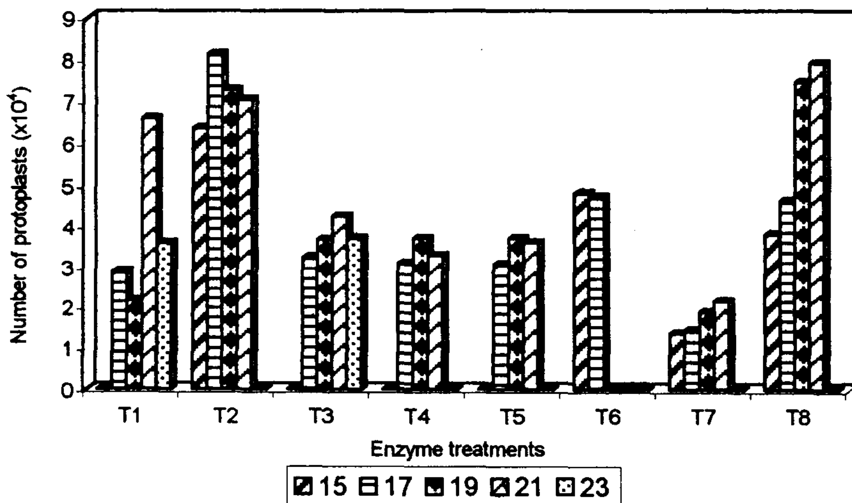
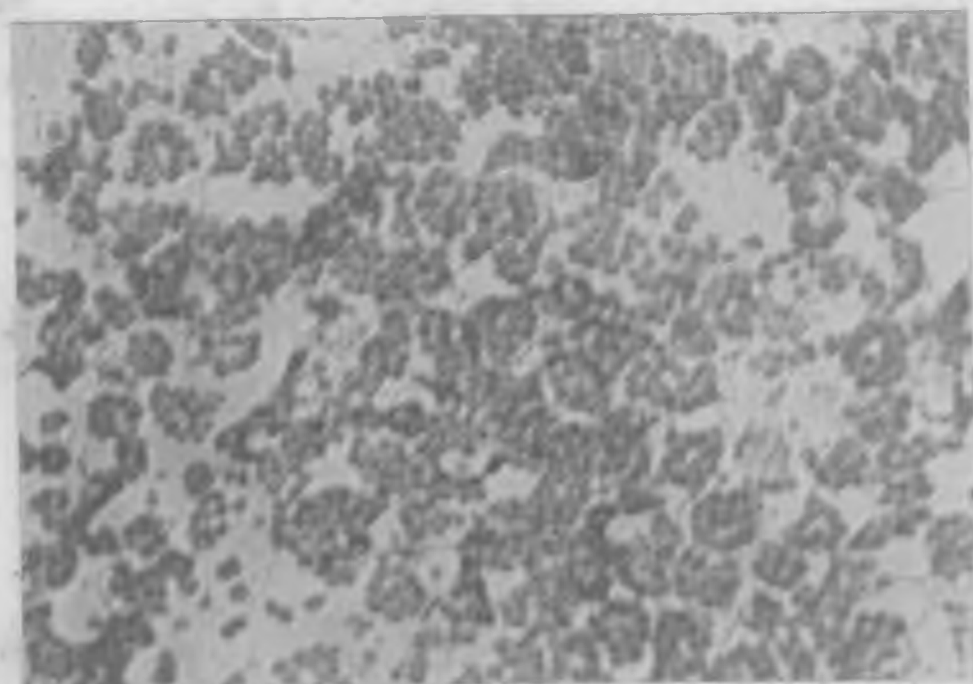
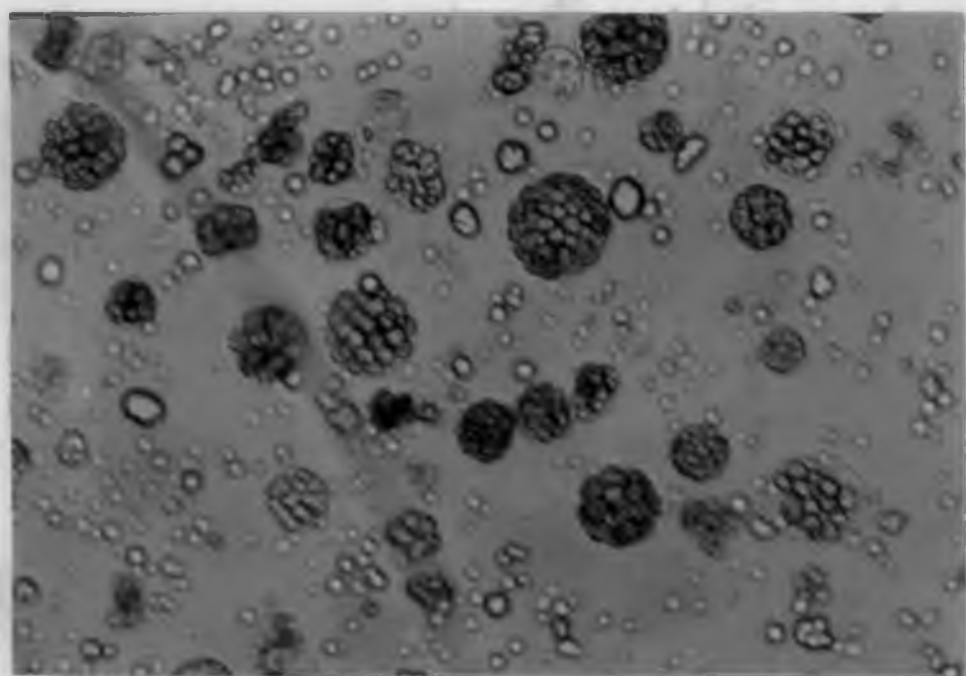


Plate 6. Interconnections between cells of *Piper colubrinum*
(T_1, 15 h, 0.6 M) (100 x)

Plate 7. Isolated protoplasts of *Piper colubrinum*
(T_2, 21 h, 0.6 M) (200 x)





treatment registered the highest protoplast isolation efficiency among all the enzyme combinations tested at 0.6 M. High value for protoplast density was accounted with T_3 (5.54×10^4) during 23 h of incubation (Plate 8).

4.7.2 Influence of enzymes and duration of incubation on viability of protoplasts at 0.6 M

During 15 h of incubation, T_8 was endorsed with higher protoplast viability (97.21%) (Table 17). The enzyme treatment T_4 (98.55%) registered higher rate of viability at 17 h of digestion. This enzyme combination recorded the highest viability among all the enzyme treatments. Viability of protoplast had shown high value in T_4 at 19 h (98.17%) and 21 h (98.48%) of incubation. At 23 h of digestion 97.36 per cent viability of protoplast was accounted for T_1 treatment.

4.7.3 Influence of enzymes and duration of incubation on yield of protoplasts at 0.65 M

The enzyme treatment T_2 recorded higher protoplast density at 15 h (6.37×10^4) and 17 h (8.12×10^4) (Plate 9) of incubation (Table 18). This enzyme treatment at 17 h registered highest protoplast isolation efficiency among all the enzyme combinations tested at 0.65 M. Higher protoplast densities were accounted with T_8 at 19 h (7.46×10^4) and 21 h (7.91×10^4) of incubation. The enzyme combination T_3 (3.71×10^4) was elicited with high yield of protoplasts during 23 h.

4.7.4 Influence of enzymes and duration of incubation on viability of protoplasts at 0.65 M

During 15 h of incubation, T_8 touched the high value of protoplast viability (96.48%) (Table 19). The enzyme treatment T_5 ranked with higher viability at 17 h (98.72 %), 19 h (98.63%) and 21 h (98.07%) of incubation. The T_5 enzyme combination at 17 h recorded the highest viability among all the enzymatic concentrations. Protoplast viability had shown moderate value of 97.62 per cent at 23 h of digestion in T_3.

Table 17. Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper colubrinum* at 0.6 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
T_1	-	98.28	97.12	97.35	97.36	78.02
T_2	94.74	92.76	93.51	92.52	-	74.71
T_3	-	97.41	97.18	96.18	96.75	77.50
T_4	-	98.55	98.17	98.48	-	59.04
T_5	-	98.42	97.14	97.63	-	58.64
T_6	91.67	90.47	-	-	-	36.42
T_7	91.57	91.24	90.52	90.06	-	72.68
T_8	97.21	96.44	97.57	96.84	-	77.61
Mean	46.90	95.44	83.90	83.63	24.26	66.82

Table 18. Influence of enzymes and duration of incubation on protoplast yield ($x10^4$) of *Piper colubrinum* at 0.65 M

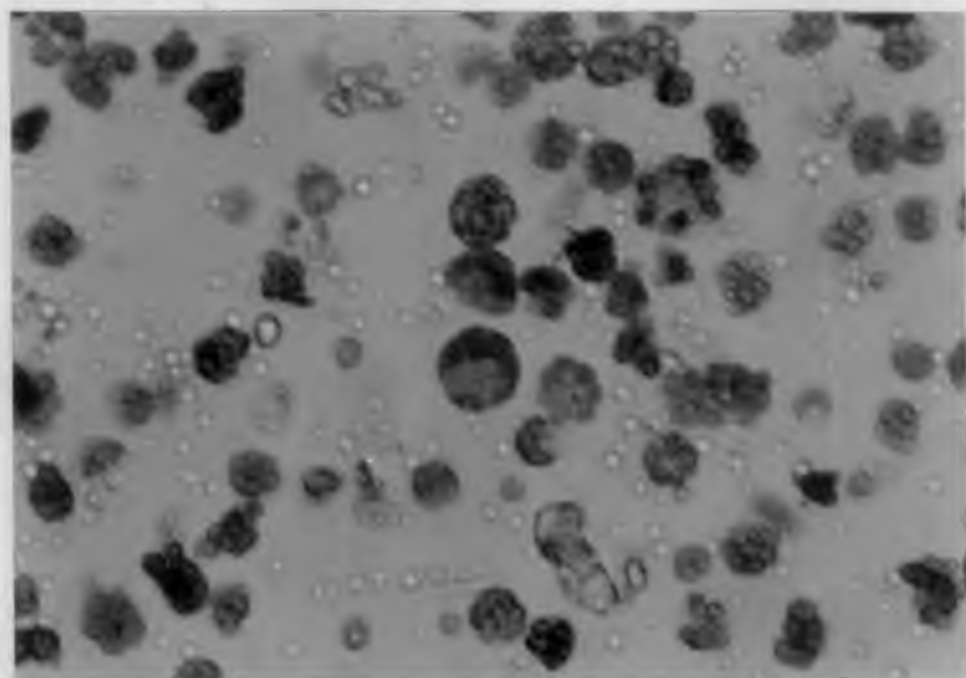
Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
T_1	-	2.90	2.16	6.60	3.59	3.05
T_2	6.37	8.12	7.27	7.04	-	5.76
T_3	-	3.24	3.68	4.24	3.71	2.97
T_4	-	3.10	3.70	3.29	-	2.02
T_5	-	3.07	3.71	3.60	-	2.08
T_6	4.80	4.71	-	-	-	1.90
T_7	1.35	1.43	1.87	2.15	-	1.36
T_8	3.81	4.62	7.46	7.91	-	4.76
Mean	2.04	3.90	3.73	4.35	0.91	2.99

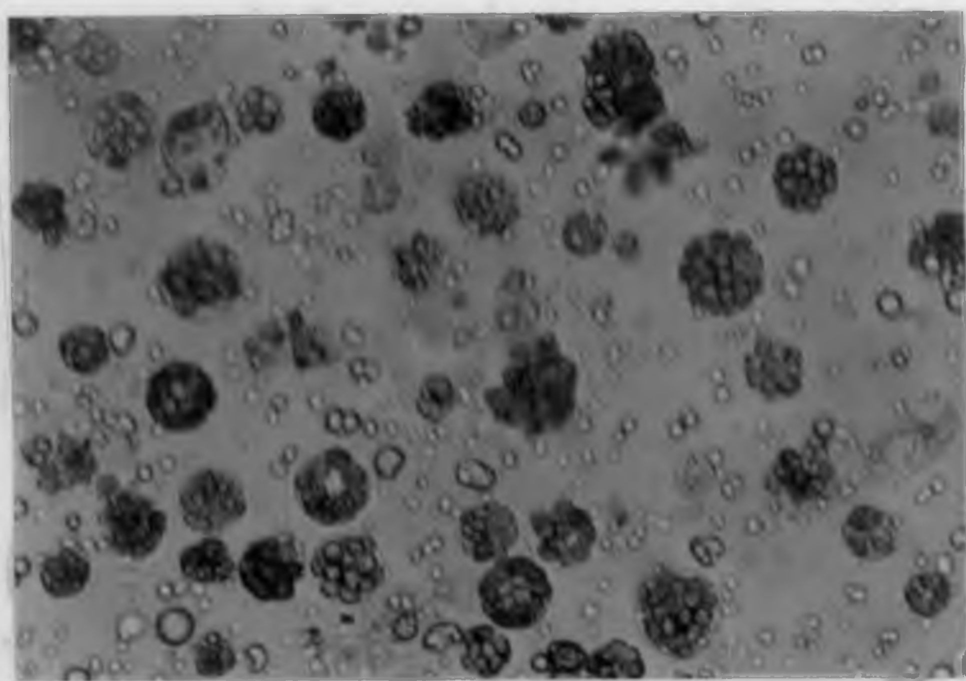
Table 19. Influence of enzymes and duration of incubation on protoplast viability (%) of *Piper colubrinum* at 0.65 M

Enzymes	Duration of incubation (h)					Mean
	15	17	19	21	23	
T_1	-	98.19	97.54	97.78	96.15	77.93
T_2	94.17	92.53	92.18	92.11	-	74.20
T_3	-	97.81	98.29	97.14	97.62	78.17
T_4	-	98.18	97.46	97.23	-	58.57
T_5	-	98.72	98.63	98.07	-	59.08
T_6	91.74	91.15	-	-	-	36.57
T_7	90.58	90.74	90.81	90.27	-	72.48
T_8	96.48	97.21	95.14	95.27	-	76.82
Mean	46.62	95.56	83.75	83.48	24.22	66.73

Plate 8. Isolated protoplasts of *Piper colubrinum* (T_3, 23 h, 0.6 M) (100 x)

Plate 9. Isolated protoplasts of *Piper colubrinum* (T_2, 17 h, 0.65 M) (200 x)





4.8 Purification of protoplasts

4.8.1 Influence of different centrifugal speed and duration on protoplast yield

The influence of speed and duration of centrifugation on protoplast yield is given in Table 20. The data revealed that centrifugation speed of 600 rpm for three minutes was found to be superior with the yield of 5.98 x 10^4 protoplasts ml^{-1}. When centrifuged at 500 rpm, some protoplasts were observed to be floating on the osmoticum. Higher speed and more duration of centrifugation resulted in disruption of the protoplasts.

4.8.2 Influence of density gradient on purification of protoplasts

During centrifugation, protoplasts formed a band at the interphase between the sucrose solution and osmoticum. However, some protoplasts pelleted at the bottom of the centrifuge tube. When protoplasts from these two gradients were examined under the microscope, intact as well as ruptured protoplasts were seen both at interphase as well as bottom pellet.

Comparatively, high yields of protoplasts were obtained at the interphase of the two solutions compared to that in the pellet (Table 21). Among the three levels of sucrose concentrations tried, good protoplast yield was exhibited at the interphase of 15 per cent sucrose concentration (3.3 x 10^4). The control treatment performed superior to the density gradient method with highest yield of 5.12 x 10^4 protoplasts ml^{-1}.

4.9 Protoplast size

Protoplasts were not uniform in size. Protoplast size varied between 10.5 µm to 21.15 µm.

4.10 Protoplast culture

Cell wall formation and cell division of protoplasts was not observed in solid and liquid media. In all the attempts made decrease in cell viability with

Table 20. Influence of different centrifugal speed and duration on protoplast yield ($x 10^4$) of *Piper colubrinum*

Duration (min)	Speed (rpm)				Mean
	500	600	700	1000	
3	2.77	5.98	1.70	4.55	3.75
5	3.79	2.55	3.53	2.16	3.01
Mean	3.28	4.27	2.62	3.36	3.38

Table 21. Influence of density gradient on protoplast yield ($x 10^4$) of *Piper colubrinum*

Sucrose (%)	Protoplast yield ($x 10^4$) at	
	Interphase	Pellet
12	2.80	1.10
15	3.30	1.00
20	2.80	1.30
Control	-	5.12

Table 22. Survival of protoplasts during culture

Days after culturing	Protoplast viability (%)	
	P. nigrum	*P.colubrinum*
0	97.48	98.48
6	67.15	56.93
12	0	0

increase in age of cultures was noticed. By the end of second week, cent per cent loss in viability was recorded in all the trials (Table 22).

Before culturing, purified *P. nigrum* protoplasts had a viability of 97.48 per cent, while *P. colubrinum* protoplasts showed 98.48 per cent viability. On the seventh day of culturing viability was found to be reduced to 67.15 per cent and 56.93 per cent in *P. nigrum* and *P. colubrinum* protoplasts respectively. By twelfth day all the protoplasts lost its viability in both the species.

4.11 Protoplast fusion

Fusion of protoplasts was not observed after 20, 30 and 40 minutes of incubation in the PEG solution. Protoplasts remained as such before and after PEG treatment.

DISCUSSION

171654

DISCUSSION

Genetic improvement of perennial species can benefit from somatic cell techniques by shortening the time for the introgression of foreign genes. Non existence of cultivar level tolerance or resistance against foot rot disease in black pepper necessitated the incorporation of incompatible wild relatives through parasexual hybridization.

The results obtained in the study "Parasexual hybridization of *Piper nigrum* and *Piper colubrinum* through protoplast fusion" are discussed in this chapter.

5.1 Enzymes

The yield of *P. nigrum* and *P. colubrinum* protoplasts varied with the concentration and ratio of the cell wall degrading enzymes. Proper enzyme combination at optimum concentration was found to influence the yield of protoplasts. In *P. nigrum*, highest yield (6.9×10^4 protoplasts ml^{-1}) was obtained at cellulase 1.4 per cent and pectinase 0.34 per cent (E_8) after 21 h of incubation while in *P. colubrinum*, cellulase 1.0 per cent along with pectinase 0.217 per cent (T_2) gave the highest yield of 13×10^4 protoplasts ml^{-1} after 21 h of incubation. The other enzyme combinations tried were less efficient. The nature and concentration of enzymes have been reported to be critical factors in the isolation of protoplasts. The best enzyme combination for protoplast isolation in tobacco suspension cultured cells was 1.0 per cent cellulase Onozuka and 0.2 per cent macerozyme (Uchimiya and Murashige, 1974), 1.0 per cent cellulase Onozuka R-10, 0.5 per cent driselase for *Pinus pinaster* (David and David, 1979), 1.0 per cent cellulysin and 0.1 per cent macerase for pea seedlings (Kaerlas *et al.*, 1992), 1.5 per cent cellulase Onozuka R-10 and 0.5 per cent macerozyme R-10 for *Actinidia* spp. leaves (Xiao and Hirsch, 1996), 1.0 per cent cellulase and 0.5 per cent pectinase for *P. nigrum* and *P. colubrinum* leaves (Philip *et al.*, 1998), 2.0 per

cent cellulase and 0.5 per cent macerozyme for *Bupleurum falcatum* suspension cultures (Bang *et al.*, 1999) and 0.5 per cent cellulase Onozuka RS and 0.05 per cent pectolyase Y 23 for *Moricanda nitens* mesophyll cells (Tian and Meng, 1999).

A lower concentration of enzyme mixture may not be able to digest off the cell wall and results in poor yield of protoplasts. Enzyme combinations, E_3, E_4 and E_6 resulted in low yield of protoplasts in *P. nigrum*. Uchimiya and Murashige (1974) reported that 1.0 per cent cellulase and 0.2 per cent macerozyme was optimum for the isolation of protoplasts from suspension cultures of tobacco. They observed that lower concentration of enzymes (0.3% cellulase and 0.1 per cent macerozyme) was inadequate and resulted in low yield of protoplasts. Ara *et al.* (2000) observed that in mango cv. Amrapali, low protoplast yield was obtained with enzyme solution, which contained lower concentrations of cellulase and pectinase.

When lower concentration (weak) of enzymes were used, isolation started only after 17 h of incubation, but when high concentration (strong) of enzymes were used, isolation was obtained at 15 h. Smith and McCown (1982) reported that in the isolation of protoplasts from *Betula* and *Rhododendron*, when weak enzymes (0.1% macerase and 0.5% cellulysin) were used, tissues were incubated for 16 to 18 h but when strong enzymes (0.5% macerease and 2.0% cellulysin) were used, isolation was obtained within four to six hours.

Higher concentration of enzyme mixture leads to excessive digestion of cell wall, resulting in subsequent bursting of protoplasts and poor yield. In *P. nigrum*, E_5 and in *P. colubrinum*, enzyme combinations T_6 and T_7 resulted in poor yield due to bursting of protoplasts. This is in support with the views of Uchimiya and Murashige (1974) and Ara *et al.* (2000) that the higher concentration of enzymes appeared excessive and resulted in poor yield of protoplasts in tobacco and mango respectively.

5.2 Osmoticum

Osmotic potential of the isolation medium is a critical factor in protoplast culture. Osmoticum like sorbitol and mannitol in the isolation medium functions as isotonic agents and provide the same mechanical pressure of the removed cell wall. This will prevent bursting of the freshly isolated protoplast. Optimum concentration of osmoticum is important, as a higher or lower concentration will lead to shrinking or bursting of protoplasts. Yield of *P. nigrum* and *P. colubrinum* protoplasts was found to vary with the level of osmoticum tried, ranging from 0.4 to 0.7 M.

In *P. nigrum*, among the six levels of osmoticum tried, maximum yield (6.5×10^4 protoplasts ml^{-1}) was recorded at 0.6 M osmoticum along with the enzyme combination E_1 during 19 h of incubation. The mean yield of protoplasts was higher at 0.6M (4.03×10^4) followed by 2.2×10^4 protoplasts ml^{-1} at 0.65M. Lowest yield of 0.74×10^4 protoplasts ml^{-1} has been obtained at 0.4 M osmotic potential with enzyme combination of 1.0 per cent cellulase and 0.28 per cent pectinase (E_1) during 17 h of digestion.

Among the six levels of osmoticum tried, in *P. colubrinum*, highest yield of protoplasts (7.7×10^4) was observed at 0.6 M osmoticum with the enzyme combination T_1 immediately after 20 h of incubation. The mean yield of protoplasts was higher at 0.6 M (4.61×10^4) followed by 0.65 M (4.19×10^4). The lowest yield (1.6×10^4) was recorded when 0.7 M osmoticum was used at cellulase 1.0 per cent and pectinase 0.186 per cent (T_1) during 22 h of incubation.

The ideal concentration of osmoticum has been reported to vary with the plant species. The best osmoticum level for the isolation of protoplasts of *Vicia hajastana* cell suspensions, barley and pea leaves were 0.7 M (Kao and Michayluk, 1974), 0.6 M for *Quercus serrata* embryogenic cultures (Susamoto and Hosoi, 1992), 0.6 M mannitol for durum wheat suspension cultures (Yang *et al.*, 1993),

0.5 M mannitol for *Allium cepa* leaves (Karim and Adachi, 1997), 10 per cent sorbitol for *Piper nigrum* and 8 per cent sorbitol for *P. colubrinum* (Philip *et al.*, 1998) and 0.4 M surcorse for *Moricanda nitens* leaves (Tian and Meng, 1999).

5.3 Incubation time

Duration of incubation of the explants in enzyme is an important factor that influences the quantity and quality of protoplasts released. The yield of *Piper* protoplasts varied with the duration of incubation in all the enzyme combinations tried. In *P. nigrum* maximum yield (6.9 x 10^4 protoplasts ml^{-1}) was recorded during 21 h of enzymatic digestion in E_8. In *P. colubrinum* also highest yield (13 x 10^4 protoplasts ml^{-1}) was observed after 21 h of incubation in T_2. The optimum duration of incubation has been reported to be influenced by the species, nature of starting material and enzymes employed. Many workers reported varying incubation time for effective isolation of protoplasts. Overnight incubation was required to isolate protoplasts from *Alnus glutinosa* (Huhtinen *et al.*, 1982), *Salix* spp. (Butt, 1985) and *Brassica nigra* hypocotyls (Narasimhulu *et al.*, 1993). Incubation duration of 24 h for banana suspension cultures (Panis *et al.*, 1993), 18 to 20 h for *Brassica campestris* cotyledonary protopasts (Zhao *et al.*, 1994), 15 h for *Piper nigrum* and *P. colubrinum* leaves (Philip *et al.*, 1998), 20 h of incubation for *Nicotiana africana* cell suspensions (Tican and Menczel, 1998) and 12 h for *Moricanda nitens* leaves (Tian and Meng, 1999).

5.4 Purification of protoplasts

Enzyme treatment resulted in a mixture of undigested cells, components of broken or burst cells and protoplasts. The mixture was partially purified to eliminate broken or undigested cells. For purifying *P. nigrum* protoplasts 1000 rpm for three minutes and for *P. colubrinum* protoplasts, 600 rpm for three minutes was found to be optimum. Higher speed and more time resulted in disruption of protoplasts in *P. colubrinum* due to fragile nature of the plasma membrane. In both

the species, lower centrifugation speed did not help in pelleting the protoplasts completely as some of the protoplasts were still floating in the enzyme solution. Cell wall materials also pelleted along with the protoplasts when lower centrifugation speed was applied. In this regard varying reports are available. The best centrifugation speed and duration for purification of protoplasts from embryogenic cell lines of *Quercus serrata* was 800 rpm for three minutes (Susamoto and Hosoi, 1992), 80 g* for five minutes for rice suspension cell derived protoplasts (Yin *et al.*, 1993), 180 g* for six minutes for cotton protoplasts from suspension cells (Peeters *et al.*, 1994), 120 g* for five minutes for *Actinidia* spp. leaf protoplasts (Xiao and Hirsch, 1996), 100 g* for five minutes for *Allium cepa* mesophyll protoplasts (Karim and Adachi, 1997), 500 rpm for *Piper colubrinum* and 750 rpm for *P. nigrum* leaf protoplasts (Philip *et al.*, 1998) and 500 rpm for five minutes for *Moricanda nitens* mesophyll protoplasts (Tian and Meng, 1999).

After pelleting and washing the protoplasts with osmoticum, floatation on sucrose solution was found to be inferior in purifying the protoplast preparations. Control treatment recorded highest yield of protoplasts compared to the floatation method. Sucrose flotation resulted in low yield due to breakage of protoplasts. Among the three levels of sucrose concentrations tried, highest yield of protoplasts was obtained at 15 per cent both for *P. nigrum* and *P. colubrinum*.

Evans and Bravo (1983) reported that in some cases sucrose floatation damage the protoplasts due to osmotic shock. They observed that concentration of sucrose or sorbitol and speed of centrifugation affect the stability of fragile protoplasts. Chanabe *et al.* (1989) reported that different yields were obtained after ficoll floatation in sunflower protoplasts due to either fragilisation of the plasma membrane by some enzymatic cocktails leading to a burst of protoplasts during centrifugation process or production of protoplasts with different sedimentation properties.

During centrifugation using sucrose solution, protoplasts of both the species formed a band at the interphase of the sucrose solution and the osmoticum. At the same time, few protoplasts pelleted together with the cell wall materials at the bottom of the centrifuge tube. This is in accordance with Millam *et al.* (1991). They found that highly meristematic (and thus dense) protoplasts sink in the sucrose solution and sediment with the debris. Nymann and Wallin (1992) opined that since mesophyll protoplasts are heavy, it was very difficult to purify it. After centrifugation at different concentrations of sucrose and sucrose + percoll, these protoplasts pelleted together with the cell wall materials. Panis *et al.* (1993) reported floatation method of protoplast purification to be inferior to sieving method.

5.5 Viability

The isolation of protoplasts has little significance unless the procedure produces viable entities. Isolated protoplasts must be healthy and viable in order to undergo sustained divisions and regeneration. Protoplasts of both the species exhibited 90 to 100 per cent viability in all the enzyme combinations tried. In *P. nigrum*, maximum viability (100%) was recorded at 0.55 and 0.65 M osmoticum in Cellulase 1.0 per cent and Pectinase 0.28 per cent (E_1) during 21 hours of digestion. In *Piper colubrinum,* highest viability (100 per cent) was observed at 0.4 M osmoticum in the enzyme mixture Cellulase 1.0 per cent and Pectinase 0.186 per cent (T_1) during 18 hours of incubation. The viability depends upon osmoticum used, concentration of enzymes, digestion period etc. Susamoto and Hosoi (1992) reported 85.0 per cent viability for protoplasts from embryogenic cell lines of *Quercus serrata* and more than 90.0 per cent protoplast viability for cotton suspensions (Peeters *et al.*, 1994), cotyledonary protoplasts of *Brassica campestris* (Zhao *et al.*, 1994) and *Moricanda nitens* mesophyll protoplasts (Tian and Meng, 1999).

5.6 Protoplast culture

Piper nigrum and *P. colubrinum* protoplasts when cultured on modified MS medium with half strength of major salts and supplemented with BAP, IAA and 2,4-D at the rate of 0.5 mg l^{-1} formed no cell wall and have not undergone any division. As age of cultures advanced, decrease in protoplast viability was observed. All the protoplasts died by the second week in both the species. Thomas (1981) in potato protoplasts and Smith and McCown (1982) in Assian white birch (*Betula platyphylla* Szechuanica) reported that protoplast division occurred only in cells with dense cytoplasm rather than in chloroplast-laden mesophyll cells. They further reported that poor culturability of the protoplasts may be due to the damage incurred during digestion and isolation or in part due to low yield and consequent low plating densities. Chen and Ku (1985) found that *Musa* spp. protoplasts from the base tissue of the youngest leaf did not proceed with cell wall regeneration and survived only for 15 days. Vasil and Vasil (1987) reported that liquid cultures are not suitable for *Musa* spp. protoplasts since they tend to aggregate. In addition, it makes it difficult to determine plating efficiencies accurately. Matsumoto *et al.* (1988) observed cell wall formation and cell division of *Musa* spp. protoplasts from bracts after five days and did not report any further development of the dividing protoplasts. Peeters *et al.* (1994) observed that *Gossypium hirsutum* protoplasts from an embryogenic cell suspension culture, when cultured on liquid medium, protoplasts aggregated and formed groups of hundreds of cells. When shaken, viability of the protoplasts was totally lost after one week of culture. The cell wall regeneration was irregular and no cell division was observed.

Philip *et al.* (1998) reported cell wall formation three days after isolation in *P. nigrum* and by 24 h in *Piper colubrinum*. The microcallus formation was observed two months after isolation in *P. nigrum* and one month after isolation in *P. colubrinum*.

5.7 Protoplast size

Protoplasts of both the species were heterogenous in size. In *P. nigrum*, protoplast size varied between 8.46 μm to 23.0 μm while in *Piper colubrinum* protoplast size varied between 10.5 μm to 21.5 μm. Philip *et al.* (1998) observed that in *P. nigrum*, the isolated protoplasts showed variability in size while in *P. colubrinum*, the isolated protoplasts were similar in size. Regarding protoplast size, varying reports are available. Ochatt (1993) reported that in hybrid ornamental shrub, weigela x florida cv. Bristol Ruby, leaf mesophyll protoplasts were generally smaller in size (x = 18.0 μm dia.) than those from stem (x = 25.0 μm dia.) or root (x = 40.0 μm dia.), tissues. Scarpa *et al.* (1993) reported that *Medicago polymorpha* protoplasts were not uniform in size and mainly two classes were present, one of which had a diameter twice than the other. Xiao and Hirsch (1996) reported that in *Actinidia* spp., the purified protoplasts were heterogenous in terms of size within a genotype and between different genotypes. Karim and Adachi (1997) stated that size of purified protoplasts from mesophyll cells and suspension cultures of *Allium cepa* ranged from 20.0 μm to 25.0 μm. Bang *et al.* (1999) reported that freshly isolated protoplasts of *Bupleurum falatum* derived from embryogenic cell suspension cultures were homogenous in size (approximately 30.0 μm in dia.) and appearance, but some protoplasts were a few times larger with more dense cytoplasm. Morgan (1999) stated that the protoplasts isolated from petiole and leaf explants of *Cyclamen persicum* ranged in size from 15.0 to 50.0 μm dia., with most at the lower size end.

5.8 Protoplast fusion

Fusion was attempted using Poly Ethylene Glycol (PEG). Fusion was not observed between protoplasts after the PEG treatment. The percentage of protoplast fusion depends on the protoplast systems and the procedure employed. In fusing chlorophyll containing cotyledon protoplasts of *Medicago sativa* with cell suspension protoplasts of *M. falcata*, Gilmour (1986) obtained values of 1.5, 3.3 and 2.6 per cent following the use of large-scale chemical fusion (density of

protoplasts adjusted to 1.0 x 10^5 ml^{-1} and 4.0 ml aliqouts of each parter mixed), small scale chemical fusion (density of each preparation adjusted to 5.0 x 10^5 ml^{-1} and 200 µl of each solution mixed) and electrofusion respectively.

Honakanen and Ryoppy (1989) observed that in PEG treated *Trifolium* spp. protoplast fusion frequencies were below one per cent. Hanson *et al.* (1989) observed fusion frequencies up to 25.0 per cent in a somatic hybridization experiment in *Lycopersicon* spp.

SUMMARY

SUMMARY

The study entitled "Parasexual hybridization of *Piper nigrum* and *Piper colubrinum* through protoplast fusion" was carried out during September 1998 to April 2000 at the Department of Plantation Crops and Spices and the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara. The salient features of the study are summarised below.

1. In *P. nigrum*, highest yield of protoplasts (6.9 x 10^4) was recorded with 1.4 per cent cellulase and 0.34 per cent pectinase.
2. Enzymes, cellulase and pectinase at a concentration of 1.0 per cent and 0.217 per cent respectively recorded highest yield of protoplasts in *P. colubrinum* (13 x 10^4 protoplasts ml^{-1}).
3. Out of the different levels of osmoticum tried, 0.6 M was found to be best for maximum yield of protoplasts in both *P. nigrum* (6.9 x 10^4) and *P. colubrinum* (13 x 10^4).
4. The lowest mean yield of protoplasts was observed when the osmolarity of the enzyme mixture was 0.7 M both in *P. nigrum* (1.54 x 10^4) and *P. colubrinum* (2.7 x 10^4).
5. Highest yield was recorded in *P. nigrum* (6.9 x 10^4 protoplasts ml^{-1}) and *Piper colubrinum* (13 x 10^4 protoplasts ml^{-1}) after 21 hours of incubation.
6. Lower concentration of enzymes resulted in poor yield of protoplasts in *P. nigrum*.
7. Higher concentration of enzymes resulted in excessive breakage of protoplasts both in *Piper nigrum* and *Piper colubrinum*.
8. When lower concentration of enzymes were used, isolation started only after 17 h of incubation but when higher concentration of enzymes were used isolation was observed at 15 h of incubation.

171654

9. In *P. colubrinum*, protoplast isolation was observed from seven hour onwards in the enzyme combination 1.0 per cent cellulase and 0.217 per cent pectinase.
10. Filtration centrifugation technique was found to be superior in purifying the *Piper* protoplasts compared to sucrose floatation method.
11. Centrifugation at 1000 rpm for three minutes was found to be the optimum for purifying *P. nigrum* protoplasts.
12. For purifying the *P. colubrinum* protoplasts, 600 rpm for three minutes was found optimum.
13. Among the three levels of sucrose concentrations tried, highest yield of protoplasts was recorded at 15 per cent in both the species.
14. Cent per cent viability was noticed in *P. nigrum* at 0.55 and 0.65 M osmoticum in 1.0 per cent cellulase and 0.28 per cent pectinase during 21 h of digestion.
15. In *P. colubrinum*, cent per cent viability was observed at 0.4 M osmoticum in the enzyme mixture 1.0 per cent cellulase and 0.186 per cent pectinase during 18 h of incubation.
16. *P. nigrum* and *Piper colubrinum* protoplasts, when cultured on modified MS medium with half strength of major salts and supplemented with BAP, IAA and 2,4-D at the rate of 0.5 mg l^{-1} formed no cell wall and have not undergone any division.
17. In both the species, during protoplast culture as age of cultures advanced, protoplast viability decreased. Viability was found to be nil by the second week in both the species of *Piper*.
18. In *P. nigrum*, protoplast size varied between 8.46 μm to 23 μm.
19. In *P. colubrinum*, protoplast size varied between 10.5 μm to 21.15 μm.
20. The fusion of protoplasts was not observed after PEG treatment in the present study.

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* Originals not seen

APPENDICES

APPENDIX-I

Basal media for pernnial crop protoplast culture

Component (in mM)	Basal medium					
	MS	D_2	A-43	K_3	8P	N_6
NH_4NO_3	20.6	3.4	10	3.1	7.5	
KNO_3	18.8	14.7	19.8	24.7	18.8	28.0
$CaCl_2$ $2H_2O$	3.0	6.1	8.8	6.1	4.0	1.1
KH_2PO_4	1.25	0.6	1.5		1.25	3.0
Na_2H PO_4 H_2O			1.1			
CaH PO_4 $2H_2O$			0.3			
$MgSO_4$ $7H_2O$	1.5	3.2	1.6	1.0	1.2	0.75
$(NH_4)_2$ SO_4	1.0					
KCI			20.1		4.0	
Fe SO_4 7 H_2O	0.1	0.1	0.05	0.1		0.1
Na_2 EDTA	0.1	0.1	0.05	0.1		0.1
Sequestrene 330 Fe	0.1					
(in µM)						
$MnSO_4$ H_2O	100	22 (.$4H_2O$)	220 (.$4H_2O$)	60	20 (.$4H_2O$)	
$ZnSO_4$ $7H_2O$	30	6 (.$4H_2O$)	86 (.$4H_2O$)	7	7	5
H_3BO_3	100	32.2	32.2	48	48	26
KI	5	1.5		4.5	4.5	5
Na_2MO_4 $2H_2O$	1	0.4	2	1	1	
$CaCl_2$ $6H_2O$	0.11	0.07		0.11	0.11	
$CuSO_4$ $5H_2O$	0.1	0.06		0.1	0.1	

APPENDIX-II

Chemical composition of Murashige and Skoog (1962) medium

Constituents	Quantity
Major elements	
Ammonium nitrate	16.5 g
Potassium nitrate	19.0 g
Magnesium sulphate	3.7 g
Potassium dihydrogen phosphate	1.7 g
Calcium chloride	4.4 g
Ferrus sulphate	2.78 g
Sodium EDTA	3.74 g
Minor elements	
Boric acid	0.62 g
Manganese sulphate	2.23 g
Zinc sulphate	0.86 g
Potassium iodide	0.083 g
Sodium molybdate	0.025 g
Cobalt chloride	0.025 g
Copper sulphate	0.025 g
Organic constituents	
Nicotinic acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine HCl	200 mg
Sucrose	30.00 g
Myo-inositol	100.00 mg
Agar	6.00 g
PH	5.8-6.0

APPENDIX-III

Chemical composition of protoplast culture medium

Constituents	Quantity
Major elements	
Ammonium nitrate	-
Potassium nitrate	19.0 g
Magnesium sulphate	3.7 g
Potassium dihydrogen phosphate	1.7 g
Calcium chloride	4.4 g
Ferrus sulphate	2.78 g
Sodium EDTA	3.74 g
Minor elements	
Boric acid	0.62 g
Manganese sulphate	2.23 g
Zinc sulphate	0.86 g
Potassium iodide	0.083 g
Sodium molybdate	0.025 g
Cobalt chloride	0.025 g
Copper sulphate	0.025 g
Organic constituents	
Nicotinic acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine HCl	200 mg
Sucrose	14.00 g* 10.00 g**
Myo-inositol	2.00 g
Mannitol	8.00 g
pH	5.8-6.0

* Piper nigrum

** Piper colubrinum

PARASEXUAL HYBRIDIZATION OF *Piper nigrum* AND *Piper colubrinum* THROUGH PROTOPLAST FUSION

By
RESMI PAUL

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

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2000

ABSTRACT

Black pepper is the most important export oriented commodity and foreign exchange earner among the Indian spices. Ravages due to diseases, particularly the most devastating Phytophthora foot rot caused by *Phytophthora capsici* is one of the major constraints in the production of black pepper all over the world. *Piper colubrinum*, a wild relative of black pepper is found to be immune to foot rot disease. Non-existance of cultivar level tolerance or resistance against foot rot disease in black pepper necessitated the incorporation of incompatible wild relatives through parasexual hybridization.

This study was undertaken in the Department of Plantation Crops and Spices and the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara between September 1998 to April 2000. The cultures of *P. nigrum* and *P. colubrinum* were initiated in test tubes and were maintained at 26±2°C temperature and 60 to 80 per cent humidity.

The green leaves excised from axenic cultures of both the species were treated with cell wall degrading enzymes, cellulase and pectinase maintained at proper osmotic concentration.

In *P. nigrum* maximum yield was observed at 1.4 per cent cellulase and 0.34 per cent pectinase. Cellulase and pectinase at a concentration of 1.0 per cent and 0.217 per cent respectively recorded highest yield in *P. colubrinum*. In both the species 0.6 M osmoticum was found to be optimum to maintain the osmotic potential of the isolation solution. Highest yield of protoplasts was recorded in both the species during 21 h of digestion.

Filtration-centrifugation technique was found to be superior in purifying the *Piper* protoplasts compared to the sucrose floatation method. Centrifugation at 1000 rpm for three minutes was found to be best for purifying *P. nigrum*

protoplasts. For purifying *P. colubrinum* protoplasts, 600 rpm for three minutes was found to be optimum.

Highest viability was noticed at 0.55 M and 0.65 M osmoticum in 1.0 per cent cellulase and 0.28 per cent pectinase during 21 h of digestion in *P. nigrum*. In *P. colubrinum*, maximum viability was observed at 0.4 M osmoticum in the enzyme mixture 1.0 per cent Cellulase and 0.186 per cent Pectinase during 18 h of incubation.

Protoplasts of both the species when cultured on modified MS medium formed no cell wall and have not undergone any division. As age of the cultures advanced, protoplast viability decreased in *P. nigrum* and *P. colubrinum*. All the protoplasts died by the second week in both the species.

The protoplasts of both the species were heterogenous in terms of size. Fusion of the protoplasts was not observed after PEG treatment in the present study.