ESTABLISHMENT OF *IN VITRO* REGENERATION SYSTEMS FROM CALLUS AND PROTOPLAST IN *Capsicum frutescens* L.

by

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(2015-11-078)

THESIS

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DECLARATION

I, hereby declare that this thesis, entitled **"ESTABLISHMENT OF *IN VITRO* REGENERATION SYSTEMS FROM CALLUS AND PROTOPLAST IN *Capsicum frutescens* L."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co-workers
BA	Benzyl adenine
$CaCl_2$	Calcium chloride
CPW	Cell Protoplast washing
cm	Centimeter
CRD	Completely Randomized Design
CD	Critical difference
CM	Culture medium
°C	Degree Celsius
2,4-D	2,4- Dichlorophenoxyacetic acid
Fig.	Figure
FM	Floatation medium
g	Gram
h	Hours
IAA	Indole-3- Acetic Acid
IBA	Indole-3-Butyric Acid
kg	Kilogram
Kn	Kinetin
µg	Micro gram
µl	Micro litre
µM	Micro molar
mg	Milli gram
mg L^{-1}	Milli gram per litre
mL	Milli litre

mm	Milli metre
mM	Milli molar
min.	Minutes
M	Molar
MS	Murashige and Skoog
viz.	Namely
pH	Negative logarithm of hydrogen ions
NAA	α- Naphthalene acetic acid
ppm	parts per million
%	Per cent
RH	Relative Humidity
rpm	Rotations per minute
Sl. No.	Serial number
sp.	Species
SE	Standard error
i.e.	That is
wt.	weight

Introduction

1. INTRODUCTION

Capsicum frutescens commonly called as bird chilli or 'Tabasco', belonging to the family Solanaceae, is a perennial herb with highly pungent small sized pods. The centre of origin of the species is reported to be South/ Central America. It is now distributed in tropical and subtropical regions of the world. This herb originally introduced from South America, is now cultivated throughout India, including all the districts of Kerala (Basu and De, 2004).

C. frutescens is used as edible fruit, food preservative and flavouring agent. The leaves and fruits of the species are used in Ayurveda, Unani and folk medicines to treat various disabilities (Hegde *et al.*, 2014). They are medicinally valuable in terms of hypoglycemic, hypolipidemic, antioxidant, circulatory stimulant, general tonic, analgesic, carminative, antiplasmodic, diaphoretic, antiseptic, rubefacient, haemostatic, gastric stimulant and carminative properties (Vinayaka *et al.*, 2010; Anthony *et al.*, 2013).The pungency of *C. frutescens* is due to capsaicinoids, which is a local anaesthetic and pain reliever. As a rubefacient, it improves blood flow and stimulate its circulation in rheumatic and arthritic conditions. Capsaicinoid also helps to prevent infection and is an excellent remedy for sore throat and laryngitis. Antifungal and insecticidal activities have also been confirmed in the species (Stergiopoulou *et al.*, 2008; Castillo-Sánchez *et al.*, 2010). Moreover, *C. frutescens* is reported as a source of resistance to a wide range of plant pathogens and pests (Khasana *et al.*, 2015). It is also reported to be resistant to *Chilli leaf curl virus* which is the major limiting factor in chilli production (Peter, 1998). These factors provide ample scope for hybridising it with other cultivated species of *Capsicum*.

However, conventional breeding techniques become troublesome because of constraints due to unviable crosses between the species, short span of seed viability, low germination percentage etc. (Cheng *et al.*, 2007; Ashrafuzzaman *et al.*, 2009; Aleza *et al.*, 2010). Hence, crop improvement in chilli through tissue culture and genetic engineering has become inevitable (Parveen and Islam, 2017).

Techniques *viz.* somaclonal variation, embryo culture, protoplast technology etc. can be exploited to generate useful genetic variation for crop improvement.

Callus being a dedifferentiated tissue, is a good source of genetic variability and somaclonal variation (Kala *et al.*, 2014). Unfortunately, *Capsicum sp.* is one of the most recalcitrant plant species to tissue culture. So, it is very much important to develop a protocol for callus induction and multiplication followed by regeneration in *Capsicum sp.*

Somatic hybridization *via* protoplast fusion is an important tool for the production of interspecific and intergeneric hybrids. The technique provides an efficient means of gene transfer from one species to another so as to break the crossing barriers and integration of parental nuclear and cytoplasmic genomes thereby generating novel germplasm (Singh *et al.*, 2015).

Isolation of viable protoplasts and its culture are pre-requisites for producing plants with desired traits through somatic hybridization and genetic engineering techniques in *Capsicum* sp.

Hence, the present study on "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L." has been proposed with the following objectives

- To establish callus culture from different explants in *C. frutescens*
- To establish protocol for protoplast isolation from callus/leaf mesophyll and to culture protoplast.

Review of Literature

2. REVIEW OF LITERATURE

Capsicum frutescens, the perennial chilli prevalent in south India, is a medicinally valuable vegetable cum spice crop. It is the most popular *Capsicum* species of Kerala homesteads and is an inevitable spice of Kerala cuisine. It is reported as a source of resistance to a wide range of plant pathogens (Peter, 1998) and has immense medicinal value in terms of hypoglycemic, hypolipidemic and cardioprotective properties (Anthony *et al.*, 2013). These factors provide ample scope for crop improvement by hybridizing it with other *Capsicum* species.

The cultivated *Capsicum* species include *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*. Among these, *C. annuum* is the most widely cultivated species in India. Traditional breeding programmes involving *C. frutescens* and C. *annuum* were with limited success (Suprunova *et al.*, 2010). Conventional breeding techniques become troublesome due to constraints in cross pollination behaviour of chilli, short span of seed viability, low germination percentage (Ashrafuzzaman *et al.*, 2009).

Plant tissue culture technology offers enormous scope for creation and utilization of genetic variants for the improvement of horticultural crops. Techniques *viz.* soma clonal variation, embryo culture, protoplast technology etc. can be exploited to generate useful genetic variation for crop improvement. Soma clonal variations can be obtained through callus culture. Callus being dedifferentiated from parent tissue, is a good source of genetic variability and adventitious shoot formation. Also, callus culture system offers many advantages as a model system for several biological investigations (Kala *et al.*, 2014).

Protoplast technology is an *in vitro* technique that can be exploited for crop improvement. Protoplast fusion technology is a promising tool to bring together the

genomes of species that cannot be combined sexually due to incompatibility barriers. It also enables the transfer of valuable polygenic agronomical traits like resistances from wild to crop species. Isolation of viable protoplasts and subsequent *in vitro* plant regeneration are pre-requisites for producing plants with desired traits through somatic hybridization and genetic engineering techniques.

In this chapter, literature on *in vitro* regeneration systems from callus and protoplast in various horticulture plant species has been reviewed.

2.1 ESTABLISHMENT OF CALLUS CULTURE AND ORGANOGENESIS

The genus *Capsicum* is recalcitrant to *in vitro* regeneration (Liu *et al.*, 1990). The establishment of an *in vitro* regeneration system from the callus involves selection of appropriate explant, standardization of culture media, hormonal combinations and culture conditions.

2.1.1 Callus induction and multiplication

Callus is an undifferentiated mass of tissue developed *in vitro* from a differentiated plant tissue. Callus being totipotent, the whole plant can be regenerated from these tissues (Ikeuchi *et al.*, 2013). Callus initiation involves three major criteria like selection of explants, medium and culture conditions (Rakshit *et al.*, 2008). Various types of calli are formed from a tissue. Based on the macroscopic characteristics, they can be classified as friable, compact or embryonic callus (Ikeuchi *et al.*, 2013).

2.1.1.1. Explant

The explant type has a profound influence on callus induction.

Rakshit *et al.* (2008) reported that hypocotyl (76.5 per cent) explants exhibited significantly better callusing than other parts of the fruits and the callus derived from cotyledons (36 per cent) was white and friable and showed excellent growth.

Ashrafuzzaman *et al.* (2009) stated that hypocotyls have higher callus induction and shoot formation than cotyledons in chilli.

Ray *et al.* (2011) noticed that in *Solanum melongena* stem explants (1.12 g) are comparatively more responsive for callus induction than root explants (0.48 g). The stem explants took only 8.20 days for callusing, while root explants took 10.20 days.

Rao and Sangapure (2014) observed that hypocotyl was a better candidate for maximum callus induction in *C. annuum*, though both cotyledon and hypocotyl explants excised from 10-12 days old seedlings were suitable for callus induction on MS medium supplemented with NAA at 2.00 mg L^{-1}.

According to Sherkar and Chavan (2014), the highest percent of callus formation was observed in cotyledon explants of tomato in MS medium containing BAP 2.00 mg L^{-1} and NAA 0.20 mg L^{-1}.

Jan *et al.* (2015) reported that the primary leaf explants of hybrid tomato cultivar Peto-86 had better callus induction frequency (90 per cent) compared to stem explants (85 per cent) on MS medium supplemented with 2, 4-D 4.00 mg L^{-1}, BAP 0.50 mg L^{-1}.

2.1.1.2 Plant Growth Regulators

Callus initiation, multiplication and type of callus formed depend on plant growth regulators and their combinations supplemented to the basal culture medium. According to Skoog and Miller (1957), an intermediate ratio of auxin and cytokinin promotes callus induction. *In vitro* callus induction and regeneration could be obtained by using auxins alone or in combination with cytokinin. The growing potential of meristematic cells can be maintained in medium supplemented with 2,4-D that enhances callus formation (Fatima *et al.*, 2014).

Luis *et al.* (2005) reported that radicle-side half-seed explant of *C. baccatum* developed organogenic calli within 4–5 months on semisolid MS medium supplemented with BAP 5.00 mg L^{-1} (22.2 mM), IAA 1.00 mg L^{-1} (5.7 mM), and GA 2.00 mg L^{-1} (6.1 mM) and it retained its organogenic ability for more than 3 years. Rakshit *et al.* (2008) observed that MS + NAA 2.00 mg L^{-1} or NAA + Kn 0.50 mg L^{-1} + 2,4-D 5.00 mgL^{-1} was the best medium for callus initiation in two varieties of *C. annuum*, Sujamukhi (friable callus) and Bally (solid callus). According to Ashrafuzzaman *et al.* (2009), the highest callus induction was obtained from hypocotyl in a combination of BAP 5.00 mg L^{-1} with NAA 0.10 mg L^{-1} and from cotyledon in a combination of BAP 5.00 mg L^{-1} with IAA 1.00 mg L^{-1} in chilli.

Osman *et al.* (2010) obtained highest callusing index (5.30) on hypocotyls explants of *Lycopersicon esculentum* cultured on MS medium supplemented with NAA at 0.50 mg L^{-1} followed by an index of 5.20 on NAA 0.10 mg L^{-1} in combination with BAP at 0.50 mg L^{-1}. However, MS + NAA (2.00 or 3.00 mg L^{-1}) gave the highest callusing index of 4.7 in case of cotyledon explants.

Ray *et al.* (2011) observed that stem explant recorded better callus induction 14.60 and 11.60 in MS media enriched with BAP 2.00 mg L^{-1} + NAA 0.50 mg L^{-1} and BAP 4.00 mg L^{-1} + NAA 0.50 mg L^{-1}, respectively in *Solanum melongena*.

Sakthivel and Manigandan (2011) opined that the cotyledon explants of *Lycopersicon esculentum* gave maximum callus induction in MS medium supplemented with BAP 3.00 mg L^{-1} and IAA 2.50 mg L^{-1} within seven days of culture.

Sheeba *et al.* (2013) reported maximum callus induction (80.67 percent) from leaf on MS medium containing NAA 3.00 mg L^{-1} after 20 days of incubation in *Physalis minima*. However, maximum fresh weight of the callus was reported on MS medium supplemented with IAA 2.00 mg L^{-1} and BAP 0.50 mg L^{-1}.

Fatima *et al.* (2014) confirmed maximum callus induction (83 per cent) in cotyledon leaf explants on MS medium containing 2,4-D 1.00 mg L^{-1} under light compared to dark culture conditions in *Capsicum* cultivars Sanam and Nepali. Rao and Sangapure (2014) reported maximum callus induction in *C. annuum* on MS medium supplemented with NAA 2.0 mg L^{-1} from hypocotyl explants. Mangang (2014) claimed that induction of callus from placental tissues of *C. chinense* were achieved in MS medium supplemented with 2,4-D 2.00 mg L^{-1} and Kn 0.50 mg L^{-1}. Raj *et al.* (2015) observed 91 per cent callus induction in MS medium + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} after 2 weeks of culture.

Jan *et al.* (2015) reported that the primary leaf explants of hybrid tomato (*Lycopersicon esculentum*) cultivar Peto-86 gave good quality callus (green and soft callus) with an induction frequency of 90 per cent on MS + 2, 4-D 4.00 mg L^{-1} + BAP 0.50 mg L^{-1}, and 71 per cent on MS + 2, 4-D 3.00 mg L^{-1} + BAP 1.00 mg L^{-1}. In stem

explant, callus induction frequency was the highest 85 per cent on MS + 2, 4-D 4.00 mg L^{-1} + BAP 0.50 mg L^{-1}.

According to Shah *et al.* (2015), hypocotyl explants recorded a callus induction frequency of 58 – 68 per cent in different cultivars of tomato on MS basal media containing IAA 2.00 mg L^{-1} and BAP 2.50 mg L^{-1}.

2.1.2 Callus mediated organogenesis

The regeneration of shoots from callus is influenced by various factors. These include genotype, explant type, media composition, quantity of media, concentration and combination of plant growth regulators, gelling agent, light intensity and quality, photoperiod, temperature etc.

Literature review often points to direct (adventitious) regeneration of shoots in *Capsicum* regeneration. Reports on indirect regeneration (through callus) is scarce. The reason can be attributed to diminishing regeneration potential of the callus with time (Luis *et al.*, 2005).

The organogenic calli developed from radicle-side half-seed explant of *C. baccatum* on semisolid MS supplemented with BAP 5.00 mg L^{-1} (22.20 mM), IAA 1.00 mg L^{-1} (5.70 mM), and GA 2.00 mg L^{-1} (6.10 mM), after subculture showed bud elongation and shoot development into complete plants after transfer to medium without plant growth regulators (Luis *et al.*, 2005).

Rakshit *et al.* (2008) observed better shoot induction from calli in MS media supplemented with IAA 0.50 mg L^{-1} + BAP1.00 mg L^{-1} in *C. annuum*.

According to Valadez-Bustos *et al.* (2009), plant regeneration studies in recalcitrant chilli pepper have been genotype dependent. In contrast to this, Fatima *et al.* (2014) opined that the regeneration behavior of *Capsicum* cultivars Sanam and Nepali was found to be independent of genotype and the highest shoot regeneration potential (53 per cent) was observed in cotyledon leaf explant induced calli on MS medium containing BAP 3.00 mg L^{-1} and Kn 1.00 mg L^{-1}.

Sakthivel and Manigandan (2011) stated that a regeneration frequency of 87.50 per cent and 11 shoots per callus were achieved on MS media supplemented with BAP 1.50 mg L^{-1} in tomato. The mean number of shoots decreased with increase in the concentrations of BAP above the optimal level (1.50 mg L^{-1}). Sheeba *et al.* (2013) reported the highest regeneration of 46.28 per cent in MS media containing BAP 2.00 mg L^{-1} + IAA 0.25 mg L^{-1} from the leaf derived calli of *Physalis minima,* after 42 days of incubation.

Raj *et al.* (2015) obtained plant regeneration from callus of *C. chinense* in MS medium supplemented with BA 5.00 mg L^{-1} and IAA 0.50 mg L^{-1}.

Jan *et al.* (2015) noticed a maximum of 87 per cent regeneration from primary leaf derived callus on MS + IAA 1.00 mg L^{-1} + BAP 3.00 mg L^{-1}, pH 5.7-5.8 while it was 82 per cent from stem derived callus on MS + IAA 0.5 mg L^{-1} + BAP 3 mg L^{-1} in hybrid tomato cultivar Peto-86.

In tomato, Shah *et al.* (2015) observed shoot regeneration frequency of 69.60 per cent and 65.30 per cent respectively in two cultivars, Rio Grande and Roma on MS media containing IAA 0.100 mg L^{-1}, Zeatin 1.00 mg L^{-1} and BAP 2.00 mg L^{-1}, while in the cultivar, Moneymaker, shoot regeneration frequency of 67.30 per cent was obtained on MS media with IAA 0.10 mg L^{-1} and BAP 3.00 mg L^{-1}.

2.1.3 Root induction

In vitro rooting process is influenced by mineral concentration, type and concentration of growth regulators and culture conditions. *In vitro* rooting of micro propagated shoots is often accomplished by auxin supplementation in the culture medium.

The *in vitro* rooting of *C. annuum* was confirmed on MS + IAA or IBA0.50 mg L^{-1} (Sanatombi and Sharma, 2007). The microshoots of *C. annuum* initiated root formation on MS medium supplemented with NAA 5.00 mg $L^{-1}$and Kn0.10 mg L^{-1} (Rakshit *et al.*, 2008).

According to Sakthivel and Manigandan (2011), MS medium having IBA 0.50 mg L^{-1} was the best medium for rooting of regenerated tomato shoots. Rao and Sangapure (2014) concluded that half strength MS medium fortified with IAA 0.20 mg L^{-1} resulted in maximum root induction from the regenerated shoots of *C. annuum*

Shah *et al.* (2015) claimed that MS media enriched with IAA 0.10 mg L^{-1}, Zeatin 2.00 mg L^{-1} and BAP 2.00 mg L^{-1} resulted in maximum hairy root formation (100.00, 91.72 and 93.74 per cent) in tomato cvs. Rio Grande, Moneymaker and Roma, respectively.

Parveen and Islam (2017) obtained the highest percentage of root induction in chilli variety, Bangladesh on MS medium containing IAA 3.00 µM.

2.2 STANDARDIZATION OF PROTOCOL FOR PROTOPLAST CULTURE

Protoplast is a functional plant cell devoid of cell wall. Protoplast isolation, fusion and culture is a prerequisite for protoplast fusion studies to develop somatic

hybrids. Protoplast technology also enables regeneration into whole plant, single cell cloning, ultra structural studies, isolation of cell organelles and chromosomes, isolation of mutants, membrane studies and in genetic transformation studies.

2.2.1 Isolation of Protoplasts

2.2.1.1 Explant

Explant source has always been an important parameter while considering protoplast isolation and regeneration. Different types of explants and sources include leaf mesophyll tissues (Castelblanque *et al.*, 2010) and callus from different tissues. Firoozobady and DeBoer (1986) showed that age and growth condition of the donor tissue are very important in regeneration of cell wall and achieving cell division. The most suitable source for protoplast isolation is mesophyll cells of young leaves. Roots, shoot apices, fruits, embryos, microspores, callus and suspension cultures can also be used.

Leaves from *in vitro* grown plants were always found to be more promising for protoplast isolation than those of *in vivo* plants (Pavan *et al.*, 2000). Suitable quantity of tissue must be taken as a starting material for obtaining good number of protoplasts as the final plating density of protoplasts is important for its further development.

According to Geetha *et al.* (2000), leaf mesophyll tissues yielded more number of viable protoplast than the cell suspension culture both in cardamom and ginger.

Kativat *et al.* (2012) opined that young leaves were preferable to hypocotyls in production of viable protoplasts in *Helianthus annuus*. An average young leaf gave a 1.7 fold higher protoplast yield than hypocotyls.

2.2.1.2 Enzymatic isolation

The isolation of protoplast can be achieved by mechanical or enzymatic method. Mechanical method is not extensively used as it is tedious and yields less viable protoplast. Sufficient yield of viable protoplast and minimally damaged or undamaged protoplast, makes enzymatic method a widely used technique in protoplast isolation.

The plant cell wall is made up of cellulose, hemicellulose and pectin. These cell wall components have to be degraded for the isolation of protoplast. The enzymes cellulase, hemicellulase and pectinase are used to degrade cellulose, hemicellulose and pectin respectively. During degradation pectinase degrades cell aggregates into individual cells, cellulase and hemicellulase degrades cell wall of individual cells by breaking β-1, 4 glycosidic bonds in the cellulose polymer to release glucose units (Acharya and Chaudhary, 2012). The yield and viability of protoplasts is influenced by the pH of the enzyme solution. A pH range of 4.5 – 6.0 and a temperature range of 25-30 °C are optimal for enzymatic activity.

The key factors affecting the protoplast yield and viability are enzyme concentration, incubation time and osmotic concentration. According to Guo *et al.* (2007), relatively high concentration of mannitol slightly plasmolyses the protoplasts that enables its easy digestion. The appropriate concentration of osmotic-pressure regulating agent prevents protoplasts devoid of cell wall, from bursting or shrinking.

Osmotic potential is adjusted by adding D-mannitol, sorbitol, glucose or sucrose to the enzyme mixture (Navratilova, 2004) and D-mannitol is mostly effective. Osmotic values of the environment into which the protoplasts are released are critically important. Different plant species, tissues and organs require different concentration of D-mannitol for the isolation of protoplasts (Kamnoon *et al.*, 2001;

Duquenne *et al.*, 2007). Generally, the concentration of D-mannitol is 0.23 to 0.90 M; however, protoplasts are more stable in a slightly hypotonic environment when compared to isotonic environment, as a higher osmotic potential prevents the protoplasts bursting, but can inhibit their division (Bhojwani and Razdan, 1996). Zhang *et al.* (2011) found that D-mannitol 0.52 M gave the highest viability (an average of 85.84 per cent) which was on par with D-mannitol 0.44 M in papaya. Since D-mannitol 0.44 M gave the highest yield of protoplasts, this concentration was considered as the suitable osmotic concentration for protoplasts isolation in papaya.

Shahin (1985) developed an efficient protocol for tomato protoplast isolation, culture, and plant regeneration. Protoplasts could be isolated from leaves, stems and cotyledons with a yield of up to 6. 6 x 10^6 per g tissues, when one g sample tissue was treated with 30 mL, 0.30 M enzyme combination of macerozyme 0.10 per cent (w/v), Cellulysin 0.75 per cent (w/v), TM-2 macronutrients and vitamins, polyvinyl pyrolidine (PVP-10) 1.00 per cent, MES (2-N-morpholinoethane sulfonic acid) buffer 5 µM, having a pH of 5.60, at an incubation of 12 h in a water bath shaker with 60 rpm and 28 °C. Tomato leaf protoplasts were characteristically spherical with dense chloroplasts. Cell wall formation occurred within 48 h after plating, as confirmed by plasmolysis in mannitol 0.90 M solution.

Geetha *et al.* (2000) recorded that leaf tissue of cardamom when incubated in an enzyme solution containing macerozyme R10 0.50 per cent, cellulose Onkozuka R10 2.00 per cent and mannitol 9.00 per cent for 18-20 h at 25 °C in dark resulted in a protoplast yield of 3.50 x 10^5 per g with 75.00 per cent viable cells. They also reported 2.50 x 10^5 per g with 55.00 per cent viable protoplast from leaf tissue of ginger when incubated in an enzyme combination macerozyme R10 0.50 per cent, hemicellulose 3.00 per cent and cellulose Onkozuka R10 5.00 per cent for 10 h at 15 °C followed by 6 h at 30 °C.

An enzyme combination of cellulase R-10 2.00 per cent and macerozyme R-10 0.50 per cent in sucrose 0.40 M - K3 solution was found to be effective in isolating tomato (*Solanum lycopersicon*) leaf protoplast after 12 h of incubation. A study on effect of salt stressed incubation solution (by partial replacement of mannitol with sodium chloride) on protoplast yield and viability showed that salt stress in incubation solution has no significant difference in protoplast yield up to 5 h but viability decreased by 55.00 per cent (Horvath, 2009).

Thomas (2009) observed maximum protoplast yield in *Tylophora indica*, when preplasmolysis followed enzyme treatment. Preplasmolysis with an osmotic solution consisting of mannitol 0.60 M + $CaCl_2 \cdot 2H_2O$ 0.10 per cent (w/v) with an osmolarity of 0.72 osmol per kg, with gentle shaking at 70 rpm for 4 h followed by enzyme treatment with 0.60 M, cellulase 0.30 per cent (w/v) and 0.50 per cent (w/v) each of macerozyme and hemicellulose at 27°C and 60 rpm for 12 h incubation gave a maximum yield 10.30×10^5 per g fresh weight, having 84.00 per cent and 65.00 per cent viability after 4 and 24 h, respectively.

In an experiment, conducted in *Carica papaya* by Zhang *et al.* (2011) a maximum of 1.50×10^7 protoplasts g^{-1} fresh weight (FW) of leaves having 90.00 per cent viability was obtained, when treated with an enzyme combination of 1.20 per cent cellulase R-10, 0.30 per cent, macerozyme R-10 and D-mannitol 0.44 M, maintained at pH 5.8 for an incubation period of 13 h in darkness at 26 °C temperature with a rotary speed of 60 rpm.

The protoplast isolation efficiency after preplasmolytic treatment and digestion of leaf and hypocotylin enzyme mixture consisting of cellulose Onozuka R 10 1.00 per cent and pectolyase Y-23 0.10 per cent, after 14–16 h of incubation reached on an average 3.00×10^6 and 10^6 protoplasts per g of leaf and hypocotyl tissue, respectively in carrot (Grzebelus *et al.*, 2011).

Ramulu *et al.* (2014) pointed out that *in vitro* leaf explants of medicinal herb, *Solanum surattense* when treated with a mixture of cellulase 2 per cent and macerozyme1.00 per cent yielded a maximum number of viable protoplast in 4-5 h of incubation in dark. Beyond 5 h of incubation the protoplast yield, gradually decreased and further resulted in complete shrinkage of protoplasts at 10-12 h of incubation.

Sura *et al.* (2015) developed a protocol for quick isolation of protoplasts of *C. annuum* using lower concentration of macerozyme 0.20 per cent and cellulase 1.00 per cent and obtained 5.8 x 10^5 protoplasts g^{-1}. Release of protoplast from mesophyll tissue increased upto 4½ h of incubation then declined beyond it and resulted in complete shrinkage at 8-9 h of incubation.

According to Wu *et al.* (2017), yield of 4.4 × 10^7 protoplasts g^{-1} fresh weight with 92.60 per cent viable protoplasts was achieved in cassava by treating mesophyll tissues in an enzyme solution having 1.60 per cent cellulase R-10 and 0.80 per cent macerozyme R-10 for 16 h at 25 °C in dark.

2.2.2 Testing protoplast viability

Mukhtar *et al.* (2012) used phenosafranine 0.10 per cent to estimate viability per cent in *Dalbergia sissoo*. Protoplasts exhibiting green color were regarded as viable and red stained were considered dead.

Geetha *et al.* (2000) estimated the viability of protoplast using Evans blue in cardamom and ginger. Kanchanapoom *et al.* (2001) claimed that the freshly isolated protoplasts were of spherical shape and were yellow green with Fluoresceindiacetate (FDA) fluorescence and red with chlorophyll fluorescence indicating viability after

release. Calcofluor white staining indicated that cell wall removal was complete after isolation. Thomas (2009) assessed viability of protoplast isolated from *Tylophora indica* with Fluoresceindiacetate (FDA) stain. Hovarth (2009) determined protoplast viability in tomato by staining with ten micro molar FDA solution. In *Carica papaya*, the viability of protoplasts was examined by FDA (12 µl) staining assay under B-2A illumination using an epifluorescence microscopy (Zhang *et al.*, 2011). The freshly isolated protoplasts were highly cytoplasmic and relatively uniform in size following purification. The viability of protoplasts was found to exceed 90 per cent as detected by FDA staining *Gentiana straminea* (Shi *et al.*, 2016).

2.2.3 Protoplast culture

The protoplast culture system entails protoplast-to-plant conversion. For successful cultivation of protoplasts, high viability and sufficient plating density are important. The nutritional requirements for micro/macrocalli formation and further plant regeneration from protoplasts, varies with different species and different tissues of the same species. The MS medium supplemented with osmoticum, usually a non metabolising sugar alcohol, such as mannitol, or the somewhat more soluble, sorbitol has been established as the basal medium for protoplast culture. The major growth regulators, auxins and cytokinins, supplement to the basal medium are normally essential for sustained protoplast growth. The growth requirements of protoplasts often change during culture, necessitating modification of medium composition. Media should be simple and fully defined to ensure reproducibility. The optimal density of protoplasts also influences the division of cells and the formation of microcalli.

Prakash *et al.* (1997) reported that protoplasts isolated from fully expanded leaves of 3-week-old axenic shoots of *C. annuum* when cultured in modified MS medium supplemented with mannitol 9.00 per cent, NAA 1.00 mg L^{-1}, 2,4-D 1.00 mg

L^{-1} and BAP 0.50 mg L^{-1}, resulted in divisions with a frequency ranging from 20-25 per cent. Addition of ascorbic acid and polyvinylpyrrolidone in the medium and incubation in dark, helped to overcome browning of protoplasts. They obtained microcalli from protoplasts of *C. annuum* in modified MS medium supplemented 2.00 NAA mg L^{-1} and BAP 0·50 mg L^{-1} and macrocalli in MS gelled medium containing NAA 2.00 mg L^{-1} and BAP 0.50 mg L^{-1}. In contrast, Vardi *et al.* (1982) observed auxins – cytokinin combinations to be detrimental for callus growth in protoplast culture of citrus.

Dovzhenko *et al.* (2003) reported auxin as sole hormone in basal culture medium for protoplast culture in carrot (*Dacus carota*) and *Arabidopsis thaliana*.

Isolated protoplasts commenced cell wall regeneration within a short time following introduction into culture. However, they required osmotic protection until their new primary walls could counteract the turgor pressure exerted by the cytoplasm. In some cases, gradual reduction of the osmotic pressure by diluting the culture medium with a solution of similar composition, but of reduced osmotic pressure, was found to be essential for sustaining mitotic division, leading to the formation of daughter cells and tissues (Davey *et al.*, 2005).

The density of protoplasts usually ranges between $1x10^4$ and $1x10^6$ protoplasts per mL of medium (Barsby *et al.*, 1986), for the division of cells and the formation of microcalli. If the density is too high, uniting and interconnecting of the cell colonies may occur (Navratilova, 2004).

According to Zhiming *et al.* (1994), a low plating density of 5.00×10^4 mL^{-1} was favourable for protoplast division in white mulberry (*Morus alba*) and observed division on 4^{th} day after culture, and the division frequency 24 per cent on 10^{th} day. A number of cell colonies and microcalli formed in 6 weeks. The microcalli were

transferred onto MSB (containing MS minerals and B5 organic components) medium with NAA 0.50 mg $L^{-1}$and BA 0.50 mg L^{-1} for further proliferation. Shoot initiated when the calli of 3-4 mm in size were transferred onto MSB differentiation medium with NAA 0.10 mg L^{-1} and BA 1.00 mgL^{-1}. The frequency of shoot formation was 35.00 per cent.

Bharadwaj *et al.* (2013) noticed cell wall regeneration at 24 h and cell division within 5 days, at a plating density of 1.00 x 10^5 protoplasts $mL^{-1}$of culture in Jute, *Corchorus capsularis*. Moreover, they observed sustained divisions after two days in medium containing 2, 4-D 1.00 mg L^{-1}, NAA 0.10 mg $L^{-1}$and zeatin 0.05 mg L^{-1}.

When protoplasts isolated from embryogenic suspension *Triticum aestivum cv.* Hartog were incubated in a modified liquid MS medium containing half strength of the macroelements, 2,4-D 5µM and glucose 0.60 M, He *et al.* (1992) observed colony formation at frequencies ranging from 0.10 per cent to 5.00 per cent. The frequency of colonies, forming fully developed plants varied between 1.00 per cent and 25.00 per cent.

Pushyami *et al.* (2011) observed microcalli formation after 60 days of repeated dilutions with medium. But couldn't obtain regeneration even on the most responsive medium standardized for *Corchorus capsularis* for petiole explants.

Al-Maarri *et al.* (2014) isolated protoplasts from leaf tissues of *Solanum tuberosum* cv. Binella and cv. Burren. When cultured on MS with Zeatin 1.00 or 1.50 mg L^{-1}, they started dividing after 4 days in the cv. Binella, and after 5 days in the cv. Burren. When Zeatin 1.00 mg L^{-1} was used, significant differences between the two cvs. Binella and Burren was recorded with respect to maximum rate of cell colonies, 1.58 ± 0.10 per cent and 1.27 ± 0.09 per cent, respectively,

Shi *et al.* (2016) reported that embryogenic calli was produced by transferring microcalli to solid MS medium containing a reduced concentration of 2,4-D (0.50 mg L^{-1}).

Materials and Methods

3. MATERIALS AND METHODS

The present study, "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L." was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The study was aimed at the establishment of callus culture from different explants and standardization of protocol for protoplast isolation from callus/leaf mesophyll and protoplast culture in *C. frutescens*.

The study was carried out in two phases *viz*., establishment of callus culture and organogenesis; and protoplast isolation and culture.

The details of materials and methods adopted for the study are described in this chapter.

3.1 PHASE I: ESTABLISHMENT OF CALLUS CULTURE AND ORGANOGENESIS

3.1.1 Culture media preparation and sterilization.

The basal culture medium used in the phase 1 study was Murashige and Skoog (Murashige and Skoog, 1962) medium. The chemicals used for media preparation were of analytical grade from Merck (Mumbai) and Himedia Laboratories Pvt. Ltd (Mumbai). Standard procedures were followed for the preparation of basal medium (Thorpe, 1980). The required quantities of chemicals were weighed out using weighing balance (Model Shimadzu AUX 120) and were dissolved in specific volume of double distilled water for preparing the stock solutions of major and minor nutrients and organic supplements. The stock solutions of growth regulators were prepared by dissolving the required amount of growth regulators in one or two drops of appropriate solvents *viz*., ethyl alcohol 50 per cent

or HCl 0.10 N or NaOH 0.10 N and made up using double distilled water in a volumetric flask. All the stock solutions were stored under refrigerated conditions (4°C). The glassware and tools used for the experiment were immersed in dilute liquid detergent (Labolene), washed thoroughly, followed by rinsing with double distilled water. After drying in hot air oven (160 – 180°C), they were autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 45 min. For media preparation, specific quantities of stock solutions were pipetted out into 1000 mL beaker containing 400 mL of double distilled water. Sucrose 30 g and inositol 100 mg were added afresh to the beaker and dissolved by constant stirring. The pH of the medium was adjusted to 5.7 by using NaOH 1 N or HCl 1 N with the aid of an electronic pH meter (Susima MP -1 PLUS). The final volume was made up to 1000 mL in a volumetric flask. Agar (Merck) 6.3 g was added to it, and was melted in a microwave oven (LG, Intellowave). The molten medium (40 mL) was dispersed into presterilized culture bottles (300 mL). The culture bottles were closed tightly with autoclavable plastic lids and sterilized at a temperature of 121°C and 1.06 kg cm^{-2} pressure for 20 min in STER 1 horizontal cylindrical autoclave (Yorko, India).

3.1.2. Establishment of callus culture

The study was conducted using *C. frutescens* variety Vellayani Samrudhi (Plate 1) procured from the Department of Olericulture, College of Agriculture, Vellayani.

3.1.2.1 Explant

Seeds of *C. frutescens* variety Vellayani Samrudhi were soaked overnight in distilled water. Surface sterilization of seeds was done using sodium hypochlorite 4.00 per cent solution for 5 min. Surface sterilized seeds were inoculated on to hormone free MS (Murashige and Skoog, 1962) medium. One month old *in vitro*

A

B C

Plate 1. *Capsicum frutescens* variety Vellayani Samrudhi A) Plant B) Flower C) Fruits

established seedlings were used as the source of explants for callus initiation. Leaf bits (*ca.* 3.00 cm^2) and internodal segments (*ca.* 1.50 cm long) excised from the seedlings were used as explants for callus initiation.

3.1.2.2 Culture media for callus induction and multiplication

The basal culture medium used for callus initiation was MS medium. The medium was supplemented with different levels of auxins, cytokinins and their combinations. Auxins *viz.*, 2,4-D (0.50 – 2.00 mg L^{-1}), NAA (0.50 – 2.00 mg L^{-1}), IBA (0.50 – 2.00 mg L^{-1}), IAA (0.50 – 2.00 mg L^{-1}), picloram (0.50 – 2.00 mg L^{-1}), cytokinins *viz.*, BA (0.50 – 2.00 mg L^{-1}), Kn (0.50– 2.00 mg L^{-1}) and their combination supplemented MS media were tried for callus initiation and multiplication. The treatments are presented in Table 1.

Observations were recorded on callus induction per cent and days for callus initiation. Growth of the callus was assessed based on a visual rating (with score '1' to the smallest and score '4' to the largest). The mean score was expressed as growth score (poor '1', medium '2', good '3', profuse '4'). The nature of callus obtained was also recorded. The treatments were replicated three times.

3.1.3 Regeneration of shoots from callus

The calli obtained from the best treatments in the above experiments were transferred to MS medium supplemented with different plant growth hormones for regeneration. The hormones tried for the study encompass auxins (NAA, IAA and IBA) at concentration ranging from 0 to 2.00 mg L^{-1} and cytokinins (BA and Kn) at concentration ranging from 1.00 to 5.00 mg L^{-1}. The treatments tried for shoot regeneration from callus are depicted in Table 2.

Table 1: Plant growth hormones tried for callus induction and multiplication

Treatments	Auxins (mg L^{-1})					Cytokinins (mg L^{-1})	
	NAA	IAA	IBA	2,4-D	Picloram	BA	Kn
C1	0.5	-	-	-	-	-	-
C2	1.0	-	-	-	-	-	-
C3	1.5	-	-	-	-	-	-
C4	2.0	-	-	-	-	-	-
C5	-	0.5	-	-	-	-	-
C6	-	1.0	-	-	-	-	-
C7	-	1.5	-	-	-	-	-
C8	-	2.0	-	-	-	-	-
C9	-	-	0.5	-	-	-	-
C10	-	-	1.0	-	-	-	-
C11	-	-	1.5	-	-	-	-
C12	-	-	2.0	-	-	-	-
C13	-	-	-	0.5	-	-	-
C14	-	-	-	1.0	-	-	-
C15	-	-	-	1.5	-	-	-
C16	-	-	-	2.0	-	-	-
C17	-	-	-	-	-	0.5	-
C18	-	-	-	-	-	1.0	-
C19	-	-	-	-	-	1.5	-
C20	-	-	-	-	-	2.0	-
C21	-	-	-	-	-	-	0.5
C22	-	-	-	-	-	-	1.0
C23	-	-	-	-	-	-	1.5
C24	-	-	-	-	-	-	2.0
C25	1.0	-	-	-	-	2.0	-
C26	-	1.0	-	-	-	2.0	-
C27	-	-	1.0	-	-	2.0	-
C28	-	-	-	1.0	-	2.0	-
C29	1.0	-	-	-	-	3.0	-
C30	-	1.0	-	-	-	3.0	-
C31	-	-	1.0	-	-	3.0	-
C32	-	-	-	1.0	-	3.0	-
C33	1.0	-	-	-	-	-	2.0
C34	-	1.0	-	-	-	-	2.0
C35	-	-	1.0	-	-	-	2.0
C36	-	-	-	1.0	-	-	2.0
C37	1.0	-	-	-	-	-	3.0
C38	-	1.0	-	-	-	-	3.0
C39	-	-	1.0	-	-	-	3.0
C40	-	-	-	1.0	-	-	3.0
C41	-	-	-	-	0.5	-	-
C42	-	-	-	-	1.0	-	-
C43	-	-	-	-	1.5	-	-
C44	-	-	-	-	2.0	-	-

Table 2: Plant growth hormones tried for shoot regeneration from callus.

Treatments	Cytokinins (mg L^{-1})		Auxins (mg L^{-1})		
	BA	Kn	NAA	IAA	IBA
R1	1.0	-	-	-	-
R2	1.0	-	0.5	-	-
R3	1.0	-	1.0	-	-
R4	1.0	-	1.5	-	-
R5	1.0	-	2.0	-	-
R6	1.0	-	-	0.5	-
R7	1.0	-	-	1.0	-
R8	1.0	-	-	1.5	-
R9	1.0	-	-	2.0	-
R10	1.0	-	-	-	0.5
R11	1.0	-	-	-	1.0
R12	1.0	-	-	-	1.5
R13	1.0	-	-	-	2.0
R14	-	1.0	-	-	-
R15	-	1.0	0.5	-	-
R16	-	1.0	1.0	-	-
R17	-	1.0	1.5	-	-
R18	-	1.0	2.	-	-
R19	-	1.0	-	0.5	-
R20	-	1.0	-	1.0	-
R21	-	1.0	-	1.5	--
R22	-	1.0	-	2.0	
R23	-	1.0	-	-	0.5
R24	-	1.0	-	-	1.0
R25	-	1.0	-	-	1.5
R26	-	1.0	-	-	2.0
R27	3.0	-	-	-	-
R28	3.0	-	0.5	-	-
R29	3.0	-	1.0	-	-
R30	3.0	-	1.5	-	-
R31	3.0	-	2.0	-	-
R32	3.0	-	-	0.5	-
R33	3.0	-	-	1.0	-
R34	3.0	-	-	1.5	-
R35	3.0	-	-	2.0	-
R36	3.0	-	-	-	0.5
R37	3.0	-	-	-	1.0
R38	3.0	-	-	-	1.5
R39	3.0	-	-	-	2.0
R40	-	3.0	-	-	-
R41	-	3.0	0.5	-	-

Treatments	Cytokinins (mg L^{-1})		Auxins (mg L^{-1})		
	BA	Kn	NAA	IAA	IBA
R42	-	3.0	1.0	-	-
R43	-	3.0	1.5	-	-
R44	-	3.0	2.0	-	-
R45	-	3.0	-	0.5	-
R46	-	3.0	-	1.0	-
R47	-	3.0	-	1.5	-
R48	-	3.0	-	2.0	-
R49	-	3.0	-	-	0.5
R50	-	3.0	-	-	1.0
R51	-	3.0	-	-	1.5
R52	-	3.0	-	-	2.0
R53	5.0	-	-	-	-
R54	5.0	-	0.5	-	-
R55	5.0	-	1.0	-	-
R56	5.0	-	1.5	-	-
R57	5.0	-	2.0	-	-
R58	5.0	-	-	0.5	-
R59	5.0	-	-	1.0	-
R60	5.0	-	-	1.5	-
R61	5.0	-	-	2.0	-
R62	5.0	-	-	-	0.5
R63	5.0	-	-	-	1.0
R64	5.0	-	-	-	1.5
R65	5.0	-	-	-	2.0
R66	-	5.0	-	-	-
R67	-	5.0	0.5	-	-
R68	-	5.0	1.0	-	-
R69	-	5.0	1.5	-	-
R70	-	5.0	2.0	-	-
R71	-	5.0	-	0.5	-
R72	-	5.0	-	1.0	-
R73	-	5.0	-	1.5	-
R74	-	5.0	-	2.0	-
R75	-	5.0	-	-	0.5
R76	-	5.0	-	-	1.0
R77	-	5.0	-	-	1.5
R78	-	5.0	-	-	2.0

Observations were recorded on days to shoot initiation, number of shoot per callus inoculated, number of nodes per shoot and shoot length. The treatments were replicated three times.

3.1.4 Rooting of *in vitro* regenerated shoots

The *in vitro* regenerated shoots were inoculated on to MS medium supplemented with different plant growth hormones. Different levels of auxins (2,4-D, NAA, IAA and IBA) at concentration ranging from 0.50 to 2.00 mg L^{-1}. The treatments tried for *in vitro* rooting of shoots are presented in Table 3.

Observations were recorded on rooting per cent, days to root initiation, number of roots per shoot and root length. The treatments were replicated three times.

All the cultures in various steps of callus culture and organogenesis were incubated in a culture room maintained at light intensity of 40 µE m^{-2} s^{-1} using white fluorescent tubes for a photoperiod of 16 h light/ 8 h dark. The temperature was regulated at 25 $\pm$ 2°C with a relative humidity of 60 per cent.

3.1.5 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant differences between treatments were determined by pairwise comparison. Each treatment was replicated three times.

3.2 PHASE II: STANDARDIZATION OF PROTOCOL FOR PROTOPLAST CULTURE

The procedure for protoplast isolation and culture is described in Plate 2.

Table 3: Plant growth hormones tried for rooting.

Treatments	Auxins (mg L^{-1})			
	NAA	IAA	IBA	2,4-D
Rt 1	0.5	-	-	-
Rt 2	1.0	-	-	-
Rt 3	1.5	-	-	-
Rt 4	2.0	-	-	-
Rt 5	-	0.5	-	-
Rt 6	-	1.0	-	-
Rt 7	-	1.5	-	-
Rt 8	-	2.0	-	-
Rt 9	-	-	0.5	-
Rt 10	-	-	1.0	-
Rt 11	-	-	1.5	-
Rt 12	-	-	2.0	-
Rt 13	-	-	-	0.5
Rt 14	-	-	-	1.0
Rt 15	-	-	-	1.5
Rt 16	-	-	-	2.0

Plate 2: Steps in protoplast isolation, purification and culture

Macerated leaves from one month old seedlings

Incubation of leaves in CPW solution with enzymes and mannitol at 25±2°C and 50 rpm shaking in dark

Filtering through cell strainer

Filtrate were collected in 15 mL centrifuge tubes and centrifuged

Pellet formed after centrifugation. Dissolved the pellet in CPW solution and centrifuged 3 times.

Pellet was dissolved in CPW 1 mL solution and layered over 9 mL floatation medium and again centrifuged (Protoplast purification)

Protoplast culture

3.2.1 Explant

Leaves from *in vitro* regenerated seedlings and calli from the experiment 3.1.2.2 were used as explants for protoplast isolation. Only *in vitro* derived explants were used so that microbial inoculum could be obviated. Leaves were cut into small strips of 2-3 mm long using sterilized scalpel blade. One gram of the macerated explant was used for protoplast isolation.

3.2.2. Protoplast isolation

The steps in protoplast isolation *viz.*, preplasmolysis and enzymatic isolation were combined for effective isolation. The digestion mixture was prepared in Cell Protoplast Washing (CPW) solution containing mannitol (0.40 to 0.80 M) maintaining a pH of 5.8. Different concentrations of cellulase "ONOZUKA R-10" from *Trichoderma viride* (1.00 - 4.00 per cent) and pectinase (0.25 – 2.00 per cent) / Macerozyme R-10 from *Rhizopus* species (0.25 - 0.50 per cent) were added to CPW solution for the preparation of digestion mixtures for protoplast isolation. Components of CPW solution is given in Table 4.

Enzyme digestion mixture was filter sterilized though cellulose nitrate filter of 0.20 micron pore size. One gram macerated leaf tissue/callus was incubated in 10 mL of enzyme mixture for varying time periods (2, 4, 6, 14 and 16 h) with a shaking of 50 rpm at 25 $\pm$ 2°C in dark. After incubation, suspension of digested tissue in the isolation mixture was sieved though 70 microns cell strainer to remove the debris. The filtrate was collected and protoplast yield and viability were assessed using haemocytometer. The different osmotica, enzyme combinations and periods of exposure tried for protoplast isolation is depicted in table 5.

Table 4: Composition of CPW solution

Serial No.	Components	Concentration (mg L^{-1})	Mg per 100 mL
1	KNO_3	101	10.10
2	$CaCl_2$ $2H_2O$	1480	148.0
3	$MgSO_4.7H_2O$	246	24.60
4	KH_2PO_4	27.2	2.72
5	KI	0.16	0.016
6	$CuSO_4.5H_2O$	0.025	0.0025
7	MES buffer	10000	1000

Table 5: Different osmotica, enzyme combinations and periods of exposure tried for protoplast isolation

Treatment	Concentration of mannitol	Enzyme combination	Time period
DM_1	0.4 M	Cellulase 1% + macerozyme0.25%	2 h
DM_2			4 h
DM_3			6 h
DM_4			14 h
DM_5			16 h
DM_6		Cellulase 2% + macerozyme0.50%	2 h
DM_7			4 h
DM_8			6 h
DM_9			14 h
DM_{10}			16 h
DM_{11}		Cellulase 4%+ macerozyme 0.25%	2 h
DM_{12}			4 h
DM_{13}			6 h
DM_{14}			14 h
DM_{15}			16 h
DM_{16}		Cellulase 4%+ macerozyme 0.50%	2 h
DM_{17}			4 h
DM_{18}			6 h
DM_{19}			14 h
DM_{20}			16 h
DM_{21}	0.5 M	Cellulase 1% + macerozyme0.25%	2 h
DM_{22}			4 h
DM_{23}			6 h
DM_{24}			14 h
DM_{25}			16 h
DM_{26}		Cellulase 2% + macerozyme0.50%	2 h
DM_{27}			4 h
DM_{28}			6 h
DM_{29}			14 h
DM_{30}			16 h
DM_{31}		Cellulase 4% + macerozyme0.25%	2 h
DM_{32}			4 h

DM_{33}			6 h
DM_{34}			14 h
DM_{35}			16 h
DM_{36}		Cellulase 4%+ macerozyme 0.50%	2 h
DM_{37}			4 h
DM_{38}			6 h
DM_{39}			14 h
DM_{40}			16 h
DM_{41}	0.6 M	Cellulase 1% + macerozyme0.25%	2 h
DM_{42}			4 h
DM_{43}			6 h
DM_{44}			14 h
DM_{45}			16 h
DM_{46}		Cellulase 2% + macerozyme0.50%	2 h
DM_{47}			4 h
DM_{48}			6 h
DM_{49}			14 h
DM_{50}			16 h
DM_{51}		Cellulase 4%+ macerozyme 0.25%	2 h
DM_{52}			4 h
DM_{53}			6 h
DM_{54}			14 h
DM_{55}			16 h
DM_{56}		Cellulase 4%+ macerozyme 0.50%	2 h
DM_{57}			4 h
DM_{58}			6 h
DM_{59}			14 h
DM_{60}			16 h

3.2.3 Protoplast yield and viability determination using haemocytometer

Haemocytometer and coverslips were wiped with 70 per cent alcohol before they were put to use. Coverslip was first affixed on to the haemocytometer. The viability of protoplast was determined by staining using the dye, phenosafranine 0.10 per cent. Stock solution was prepared by dissolving 0.01 g phenosafranine in 10 mL sterile double distilled water. Equal volumes of 0.10 per cent phenosafranine stain and protoplast suspension are added to microfuge tube and mixed well by inversion, to avoid protoplast damage. Protoplast/phenosafranine mixture (approximately 20µl) was transferred to haemocytometer along the edge of the coverslip and allowed to run under. The protoplast yield and viability percentage were determined by observing under the compound microscope (Leica). On staining with phenosafranine, the dead protoplast gave red/pink colour and the viable ones remained unstained (green/white). The viability percentage was determined by the following formula.

$$\text{Viability per cent} = \frac{\text{No. of viable protoplast x 100}}{\text{Total no. of protoplast}}$$

The yield was determined by counting the number of protoplasts per gram fresh weight of leaves/ calli.

3.2.4. Protoplast purification

3.2.4.1. Protoplast washing

Protoplasts isolated from the best treatment in the above experiments, was filtered to remove the debris. The filtrate was collected in screw capped 15 mL centrifuge tubes and centrifuged at 700-2000 rpm for 10 min for further separation of protoplasts from fine debris that remained floating. The pellet of protoplasts was

resuspended in 1.00 mL of CPW medium and the process was repeated three times to wash out all the enzymes present. The pellet was again resuspended in 1.00 mL of CPW solution and then layered on 9.00 mL of floatation medium of varying sucrose levels (15 - 25 per cent) in 15.00 mL screw capped centrifuge tubes. Components of floatation medium are given in Table 6. It was then centrifuged again at 1000 and 2000 rpm for 5 min. A clear band of protoplast was produced at the interphase of CPW and floatation medium. The interphase was recovered carefully using a Pasteur pipette and mixed with equal volume of fresh CPW medium. The yield of protoplasts and its viability percentage were determined using haemocytometer.

2.3.5. Protoplast culture.

After purification, the protoplasts were transferred to liquid culture medium I (CM I) at a plating density of 2 to 10 x 10^5 protoplasts per mL in disposable 10 mm petriplates. Then they are sealed with parafilm and were dark incubated for microcalli initiation. On microcalli initiation, liquid culture medium II (CM II) was added and incubated under diffused light for further development and visual colony formation of microcalli. Compositions of liquid culture media for protoplast culture are given in Table 7. Different levels of mannitol and 2,4-D in liquid culture medium I and plating density for microcalli initiation are presented in Table 8. Observations were made for microcalli formation.

Table 6: Composition of floatation medium

Serial No.	Components	Concentration (mg L^{-1})	Mg per 100 mL
1	KNO_3	101	10.1
2	$CaCl_2$. $2H_2O$	1480	148
3	$MgSO_4.7H_2O$	246	24.6
4	KH_2PO_4	27.2	2.72
5	KI	0.16	0.016
6	$CuSO_4$. $5H_2O$	0.025	0.0025
7	Sucrose	150000-250000	15000-25000

Table 7: Composition of liquid culture media for protoplast culture

Serial No.	Components and concentration (for 100 mL)	CM I	CM II
1.	MS Stock A	2.50 mL	2.50 mL
2.	MS Stock B	0.50 mL	0.50 mL
3.	MS Stock C	0.10 mL	0.10 mL
4.	MS Stock D	0.5 0mL	0.50mL
5.	MS Stock E	0.05 mL	0.05 mL
6.	MS Stock F	0.10 mL	0.10 mL
7.	BAP	0.05 mg	0.05 mg
8.	NAA	0.05 mg	0.20 mg
9.	2,4- D	0.05 -0.60 mg	-
10	Sucrose	3.00 g	0.50 g
11	Mannitol	7g (0.40M) – 9g (0.50 M)	6 g (0.30M)-7g (0.40 M)
12	Agar	-	-

Table 8: Different levels of mannitol and 2,4-D in liquid culture medium I and plating density for microcalli initiation.

Treatments	Composition of Liquid Culture Medium (I)	Plating density
PCM_1	L MS + mannitol 0.40 M + 2,4- D 0.50 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_2	L MS + mannitol 0.40 M + 2,4- D 2.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_3	L MS + mannitol 0.40 M + 2,4- D 4.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_4	L MS + mannitol 0.40 M + 2,4- D 6.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_5	L MS + mannitol 0.50 M + 2,4- D 0.50 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_6	L MS + mannitol 0.50 M + 2,4- D 2.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_7	L MS + mannitol 0.50 M + 2,4- D 4.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5
PCM_8	L MS + mannitol 0.50 M + 2,4- D 6.00 mg L^{-1}	2 x 10^5
		5 x 10^5
		10 x 10^5

L MS – Liquid MS medium

Results

4. RESULTS

Studies on "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L." were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The results of the investigations are presented in this chapter.

4.1 PHASE1: ESTABLISHMENT OF CALLUS CULTURE AND ORGANOGENESIS

4.1.1 Establishment of callus culture

Seeds of *C. frutescens* variety Vellayani Samrudhi commenced germination after 7 to 10 days when inoculated on to MS medium devoid of hormones. 95 per cent of seeds germinated and established well. Leaves and internodes excised from *in vitro* germinated one month old seedlings were inoculated on MS medium containing various growth hormones (0.50 mg L^{-1} – 2.00 mg L^{-1}) and hormonal combinations to study the effect on callus induction.

4.1.1.1 Callus initiation and multiplication from leaf explant

Seven different hormones (NAA, IAA, IBA, 2,4-D, picloram, BA, Kn) of varying concentration (0.50 mg L^{-1} – 2.00 mg L^{-1}) and their combinations were tried to assess their effect on callus induction. The basal culture medium used for the study was MS medium. The results of the study are presented in table 9.

Among the 44 different hormonal treatments tried, 19 treatments gave callus induction. Two levels of NAA [1.50 mg L^{-1}(C3) and 2.00 mg L^{-1} (C4)], four levels of picloram [0.50 mg L^{-1} (C41), 1.00 mg L^{-1} (C42), 1.50 mg L^{-1} (C43), 2.00 mg L^{-1} (C44)] and seven auxin- cytokinin combinations [BA 3.00 mg L^{-1}+ NAA 1.00 mg L^{-1} (C29), BA 3.00 mg L^{-1}+ IAA 1.00 mg L^{-1} (C30), BA 3.00 mg L^{-1}+ IBA 1.00 mg L^{-1} (C31), BA 3.00 mg L^{-1}+ 2,4-D 1.00 mg L^{-1} (C32), Kn 3.00 mg L^{-1}+ NAA 1.00 mg L^{-1}

Table 9: Effect of auxins and cytokinins on callus initiation and multiplication from leaf explant

Treatments	Auxins (mg L^{-1})					Cytokinins (mg L^{-1})		Callus growth parameters			
	NAA	IAA	IBA	2,4D	Pic	BA	Kn	DCI	CIP	G. S	Nature of callus
C1	0.50	-	-	-	-	-	-	-	-	-	-
C2	1.00	-	-	-	-	-	-	-	-	-	-
C3	1.50	-	-	-	-	-	-	10.42	100	4	Yellowish green soft friable callus, 58.33% root initiation
C4	2.00	-	-	-	-	-	-	9.92	100	4	Yellowish green soft friable callus, 75% root initiation
C5	-	0.50	-	-	-	-	-	-	-	-	41.6% root initiation
C6	-	1.00	-	-	-	-	-	-	-	-	50% root initiation
C7	-	1.50	-	-	-	-	-	-	-	-	91.66% root initiation
C8	-	2.00	-	-	-	-	-	-	-	-	58.33% root initiation
C9	-	-	0.50	-	-	-	-	-	-	-	-
C10	-	-	1.00	-	-	-	-	-	-	-	-
C11	-	-	1.50	-	-	-	-	-	-	-	100% root initiation
C12	-	-	2.00	-	-	-	-	-	-	-	75% root initiation
C13	-	-	-	0.50	-	-	-	-	-	-	-
C14	-	-	-	1.00	-	-	-	-	-	-	-
C15	-	-	-	1.50	-	-	-	-	-	-	-
C16	-	-	-	2.00	-	-	-	17.50	50.0	2	Hard callus
C17	-	-	-	-	-	0.50	-	-	-	-	-
C18	-	-	-	-	-	1.00	-	-	-	-	-
C19	-	-	-	-	-	1.50	-	-	-	-	-
C20	-	-	-	-	-	2.00	-	-	-	-	-
C21	-	-	-	-	-	-	0.50	-	-	-	-
C22	-	-	-	-	-	-	1.00	-	-	-	-

Basal medium: MS medium, Pic: Picloram, DCI: Days for callus induction, CIP: Callus induction percentage, G.S: Growth score. Treatments that gave CIP ≤ 25 % were omitted from statistical analysis.

Table 9 continued.....

Treatments	Auxins (mg L^{-1})					Cytokinins (mg L^{-1})		Callus growth parameters			
	NAA	IAA	IBA	2,4D	Pic	BA	Kn	DCI	CIP	G. S	Nature of callus
C23	-	-	-	-	-	-	1.50	-	-	-	-
C24	-	-	-	-	-	-	2.00	-	-	-	-
C25	1.00	-	-	-	-	2.00	-	19.80	66.66	2	Hard callus
C26	-	1.00	-	-	-	2.00	-	-	-	-	-
C27	-	-	1.00	-	-	2.00	-	-	-	-	-
C28	-	-	-	1.00	-	2.00	-	23.83	41.66	2	Hard callus
C29	1.00	-	-	-	-	3.00	-	8.75	100	3	White/ yellowish green, hard profuse callus
C30	-	1.00	-	-	-	3.00	-	16.00	100	1	White/ yellowish green hard callus. Poor callus growth
C31	-	-	1.00	-	-	3.00	-	12.50	100	1	White/ yellowish green hard callus. Poor callus growth
C32	-	-	-	1.00	-	3.00	-	12.42	100	2	White/ yellowish green hard callus. Medium callus growth
C33	1.00	-	-	-	-	-	2.00	26.67	41.66	2	Hard callus
C34	-	1.00	-	-	-	-	2.00	-	-	-	-
C35	-	-	1.00	-	-	-	2.00	-	-	-	-
C36	-	-	-	1.00	-	-	2.00	27.66	25.0	2	Hard callus
C37	1.00	-	-	-	-	-	3.00	16.33	100	2	Hard callus
C38	-	1.00	-	-	-	-	3.00	22	100	1	Hard callus
C39	-	-	1.00	-	-	-	3.00	16.86	75	1	Hard callus
C40	-	-	-	1.00	-	-	3.00	19.17	100	2	Hard callus
C41	-	-	-	-	0.50	-	-	9.25	100	3	White soft profuse callus
C42	-	-	-	-	1.00	-	-	7.08	100	4	White soft profuse callus
C43	-	-	-	-	1.50	-	-	6.83	100	4	White soft profuse callus
C44	-	-	-	-	2.00	-	-	6.92	100	4	White soft profuse callus
CD	-	-	-	-	-	-	-	1.12	-	-	-
f value	-	-	-	-	-	-	-	1.93	-	-	-

Basal medium: MS medium, Pic: Picloram, DCI: Days for callus induction, CIP: Callus induction percentage, G.S: Growth score. Treatments that gave CIP ≤ 25 % were omitted from statistical analysis.

(C37), Kn 3.00 mg L^{-1}+ IAA 1.00 mg L^{-1} (C38), Kn 3.00 mg L^{-1} + 2,4-D 1.00 mg L^{-1} (C40)] in MS medium gave 100 per cent callus induction.

NAA produced yellowish green soft friable callus with root initials. Later it became slightly brown in 45 days of culture, after which the callus commenced to multiply. Both the concentrations of NAA (C3 and C4) initiated callus in about 10 days. In C3 and C4 callus initiation occurred in 10.42 and 9.92 days respectively. Both the treatments gave profuse callusing with a growth score of '4' (Plate 3A, 3B).

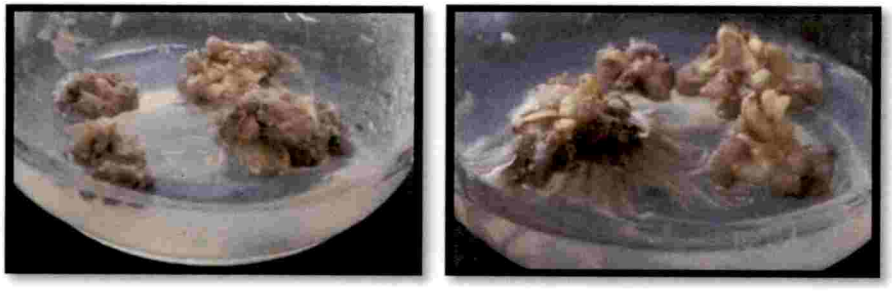
The treatments supplemented with IAA and IBA, did not initiate callus, instead root initials developed from the leaf explants (Plate 3 - C, D, E, F, G, H). Among these, two treatments C9 (MS + IBA 0.50 mg L^{-1}) and C10 (MS + IBA 1.00 mg L^{-1}) neither initiated callus nor any morphogenetic response.

Among the four 2,4-D supplemented treatments, only one responded with callusing. C16 (MS + 2,4-D 2.00 mg L^{-1}) initiated callus in 17.50 days and recorded 50 per cent callus induction with a growth score of '2' (Plate 3 I).

The picloram supplemented media exhibited profuse callusing with white soft callus. The earliest callusing (6.83 days) was observed in C43 (picloram 1.50 mg L^{-1}) which was on par with C42 (picloram 1.00 mg L^{-1}), C44 (picloram 2.00 mg L^{-1}) with a growth score of 4 in all the three treatments (Plate 4).

The eight treatments augmented with cytokinins alone, BA and Kn (C17 – C24) at various concentrations did not initiate callus or any morphogenetic response.

The treatments involving combinations of BA and auxins, earliest (8.75 days) callusing was obtained in the treatments C29. Other combination treatments of BA with auxins (IAA, IBA, 2,4-D) took more number of days to initiate callus. C30, C31, C32, gave callus initiation in 16.00, 12.50 and 12.42 days, respectively. The growth score reported in C29 was '3' but C30, C31 and C32 gave low growth score of 1, 1 and 2 respectively. Hard callus appeared to be white at initiation which later turned



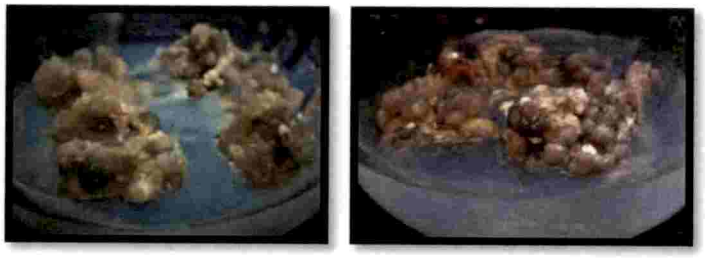
A) NAA 1.50 mg L^{-1} B) NAA 2.00 mg L^{-1}

C) IAA 0.50 mg L^{-1} D) IAA 1.00 mg L^{-1} E) IAA 1.50 mg L^{-1} F) IAA 2.00 mg L^{-1}

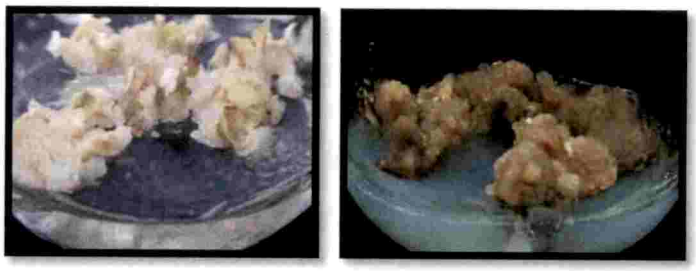
G) IBA 1.50 mg L^{-1} H) IBA 2.00 mg L^{-1} I) 2,4- D 2.00 mg L^{-1}

Plate 3: Effect of NAA, IAA, IBA and 2,4-D in MS medium on callus induction and multiplication from leaf explants 45 days after inoculation (DAI)

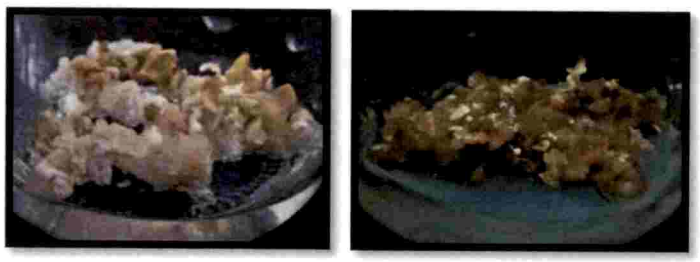
14 days after inoculation 45 days after inoculation



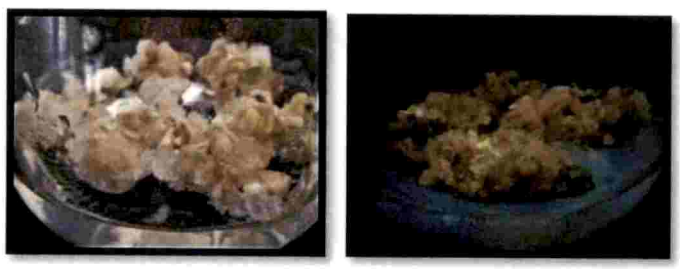
Picloram 0.50 mg L^{-1}



Picloram 1.00 mg L^{-1}



Picloram 1.50 mg L^{-1}



Picloram 2.00 mg L^{-1}

Plate 4: Effect of picloram in MS medium on callus induction and multiplication from leaf explants, 14 and 45 days after inoculation

yellowish green in all the treatments containing BA (3.00 mg L^{-1}) - auxin combinations. In those treatments with BA (2.00 mg L^{-1}) - auxin combinations, callus initiated only in treatments C25 (MS + BA 2.00 mg L^{-1} + NAA 1.00 mg L^{-1}) and C28 (MS + BA 2.00 mg L^{-1} + 2,4-D 1.00 mg L^{-1}) in 19.80 and 23.83 days, with a callus induction of 66.67 and 41.67 per cent, respectively. Hard callus with a growth score of '2' was observed in both the treatments. This callus turned dark within a week after initiation and did not give further multiplication (Plate 5).

Among the various treatments tried, those involving combinations of Kn and auxins, the earliest (16.33 days) callusing was obtained in the treatment C37, which is on par with C39 (MS + Kn 3.00 mg L^{-1} + IBA 1.00 mg L^{-1}) that took 16.86 days for callus initiation. The callus initiated late (26.67 days) in C33 (MS + Kn 2.00 mg L^{-1} + NAA 1.00 mg L^{-1}). C37, C38, C40 gave 100 per cent callus induction followed by C39 (75 per cent). Lowest (25 per cent) callus induction was obtained in C36 (MS + Kn 2.00 mgL^{-1} + 2,4-D 1.00 mgL^{-1}). C34 (MS + Kn 2.00 mgL^{-1} + IAA 1.00 mgL^{-1}) and C 35 (MS + Kn 2.00 mgL^{-1} + IBA 1.00 mgL^{-1}) did not give callus initiation. All the treatment combination involving Kn - auxins gave a growth score of '2' with yellowish green hard callus that later turned brown (Plate 6).

Among the 44 treatments tried with leaf as the explant, better callusing response with respect to days to callus initiation, callus induction per cent and growth score, was obtained with treatments supplemented with picloram (C42, C43, C44) followed by those supplemented with NAA (C3, C4) and BA - NAA combination (C29).

4.1.1.2 Callus initiation and multiplication from internodal explant

The callus initiation from the internodal explants were tried using the same treatments as with leaf explant. Among the 44 treatments tried, 100 per cent callus induction was achieved in those supplemented with two levels of NAA [1.50 mg L^{-1} (C3) and 2.00 mg L^{-1} (C4)], four levels of picloram [0.50 mg L^{-1} (C41), 1.00 mg L^{-1}

BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1} BA 3.00 mg L^{-1} + IAA 1.00 mg L^{-1}

BA 3.00 mg L^{-1} + 2,4- D 1.00 mg L^{-1} BA 2.00 mg L^{-1} + NAA 1.00 mg L^{-1} BA 2.00 mgL^{-1} + 2,4- D 1.00 mgL^{-1}

Plate 5: Effect of BA – auxin combinations in MS medium on callus induction and multiplication from leaf explants, 30 days after inoculation

Kn 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} Kn 3.00 mg L^{-1} + 2,4- D 1.00 mg L^{-1} Kn 3.00 mgL^{-1} + IAA 1.00 mgL^{-1}

Kn 3.00 mg L^{-1} + IBA 1.00 mg L^{-1} Kn 2.00 mg L^{-1} + NAA 1.00 mg L^{-1} Kn 2.00 mgL^{-1} + 2,4- D 1.00 mgL^{-1}

Plate 6: Effect of Kn – auxin combinations in MS medium on callus induction and multiplication from leaf explants, 45 days after inoculation

(C42), 1.50 mg L^{-1} (C43), 2.00 mg L^{-1} (C44)] and four BA - cytokinin combinations [BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} (C29), BA 3.00 mg L^{-1} + IAA 1.00 mg L^{-1} (C30), BA 3.00 mg L^{-1}+ IBA 1.00 mg L^{-1} (C31), BA 3.00 mg L^{-1} + 2,4-D 1.00 mg L^{-1} (C32)]. The results of the study are presented in table 10.

In NAA supplemented treatments, callus initiation occurred in 11 to 15 days, with earliest (11.25 days) callusing in C3 which was on par with C4 and with a growth score of '4' in both the treatments. Callus was initiated in 14.64 and 14.75 days, with callus induction per cent of 83.33 and 75.00 per cent and a growth score of 3 and '2' in C2 (MS + NAA 1.0 mg L^{-1}) and C1 (MS + NAA 0.5 mg L^{-1}), respectively. With all these treatments, yellowish green soft friable callus was obtained (Plate 7).

The treatments supplemented with IAA did not initiate callus. In IBA supplemented medium, C11 (MS + IBA 1.50 mg L^{-1}) and C12 (MS + IBA 2.00 mg L^{-1}) initiated callus with root initials in 19 and 18.83 days with callus induction of 66.67 and 58.33 per cent, respectively (Plate 8 - A, B). Both the treatments recorded a growth score of '2". Two treatments, C9 (MS + IBA 0.50 mg L^{-1}) and C10 (MS + IBA 1.00 mg L^{-1}) did not initiate callus.

Among the four 2,4-D supplemented treatments, only one responded with callusing. C16 (MS + 2,4-D 2.00 mg L^{-1}) initiated callus in 17.17 days and recorded 75 per cent callus induction with a growth score of '2' (Plate 8 C).

The picloram supplemented media exhibited profuse callusing with white soft callus. The earliest callusing (6.58 days) was observed in C43 (MS + picloram 1.50 mg L^{-1}) which was on par with C42 (MS + picloram 1.00 mg L^{-1}) (6.92 days), C44 (MS + picloram 2.00 mg L^{-1}) (6.75 days). C41 (MS + picloram 0.50 mg L^{-1}) initiated callus in 8.17 days. All these treatments, exhibited 100 per cent callus induction with a growth score of '4' (Plate 9).

Table: 10 Effect of auxins and cytokinins on callus initiation and multiplication from internodal segments explant

Treat ments	Auxins (mg L^{-1})					Cytokinins (mg L^{-1})		Callus growth parameters			
	NAA	IAA	IBA	2,4-D	Pic	BA	Kn	DCI	CIP	G. S	Nature of callus
C1	0.50	-	-	-	-	-	-	14.75	75.00	2	Yellowish green soft friable callus
C2	1.00	-	-	-	-	-	-	14.64	83.33	3	Yellowish green soft friable callus
C3	1.50	-	-	-	-	-	-	11.25	100	4	Yellowish green soft friable callus
C4	2.00	-	-	-	-	-	-	11.33	100	4	Yellowish green soft friable callus
C5	-	0.50	-	-	-	-	-	-	-	-	-
C6	-	1.00	-	-	-	-	-	-	-	-	-
C7	-	1.50	-	-	-	-	-	-	-	-	-
C8	-	2.00	-	-	-	-	-	-	-	-	-
C9	-	-	0.50	-	-	-	-	-	-	-	-
C10	-	-	1.00	-	-	-	-	-	-	-	-
C11	-	-	1.50	-	-	-	-	19.00	66.66	2	Soft callus with root initials
C12	-	-	2.00	-	-	-	-	18.83	58.33	2	Soft callus with root initials
C13	-	-	-	0.50	-	-	-	-	-	-	-
C14	-	-	-	1.00	-	-	-	-	-	-	-
C15	-	-	-	1.50	-	-	-	-	-	-	-
C16	-	-	-	2.00	-	-	-	17.17	75.00	2	Hard callus
C17	-	-	-	-	-	0.50	-	-	-	-	-
C18	-	-	-	-	-	1.00	-	-	-	-	-
C19	-	-	-	-	-	1.50	-	-	-	-	-
C20	-	-	-	-	-	2.00	-	-	-	-	-
C21	-	-	-	-	-	-	0.50	-	-	-	-
C22	-	-	-	-	-	-	1.00	-	-	-	-

Basal medium: MS medium, Pic: Picloram, DCI: Days for callus induction, CIP: Callus induction percentage, G.S: Growth score, Treatments that did not give callus induction are omitted from statistical analysis.

Table 10 continued....

Treat ments	Auxins (mg L^{-1})					Cytokinins (mg L^{-1})		Callus growth parameters			
	NAA	IAA	IBA	2,4-D	Pic	BA	Kn	DCI	CIP	G. S	Nature of callus
C23	-	-	-	-	-	-	1.50	-	-	-	-
C24	-	-	-	-	-	-	2.00	-	-	-	-
C25	1.00	-	-	-	-	2.00	-	-	-	-	-
C26	-	1.00	-	-	-	2.00	-	-	-	-	-
C27	-	-	1.00	-	-	2.00	-	-	-	-	-
C28	-	-	-	1.00	-	2.00	-	-	-	-	-
C29	1.00	-	-	-	-	3.00	-	9.17	100	3	Hard callus
C30	-	1.00	-	-	-	3.00	-	12.75	100	3	Hard callus
C31	-	-	1.00	-	-	3.00	-	11.25	100	3	Hard callus
C32	-	-	-	1.00	-	3.00	-	11.92	100	3	Hard callus
C33	1.00	-	-	-	-	-	2.00	-	-	-	-
C34	-	1.00	-	-	-	-	2.00	-	-	-	-
C35	-	-	1.00	-	-	-	2.00	-	-	-	-
C36	-	-	-	1.00	-	-	2.00	-	-	-	-
C37	1.00	-	-	-	-	-	3.00	16.78	75.00	1	Hard callus
C38	-	1.00	-	-	-	-	3.00	-	-	-	-
C39	-	-	1.00	-	-	-	3.00	-	-	-	-
C40	-	-	-	1.00	-	-	3.00	19.67	75.00	1	Hard callus
C41	-	-	-	-	0.50	-	-	8.17	100	4	Soft white profuse callus
C42	-	-	-	-	1.00	-	-	6.92	100	4	Soft white profuse callus
C43	-	-	-	-	1.50	-	-	6.58	100	4	Soft white profuse callus
C44	-	-	-	-	2.00	-	-	6.75	100	4	Soft white profuse callus
CD	-	-	-	-	-	-	-	1.17	-	-	-
f value	-	-	-	-	-	-	-	1.97	-	-	-

Basal medium: MS medium, Pic: Picloram, DCI: Days for callus induction, CIP: Callus induction percentage, G.S: Growth score, Treatments that did not give callus induction are omitted from statistical analysis.

NAA 0.50 mg L^{-1}(15DAI)

NAA 1.00 mg L^{-1}(15DAI)

NAA 1.50 mg L^{-1} (15DAI)

NAA 1.50 mg L^{-1} (45DAI)

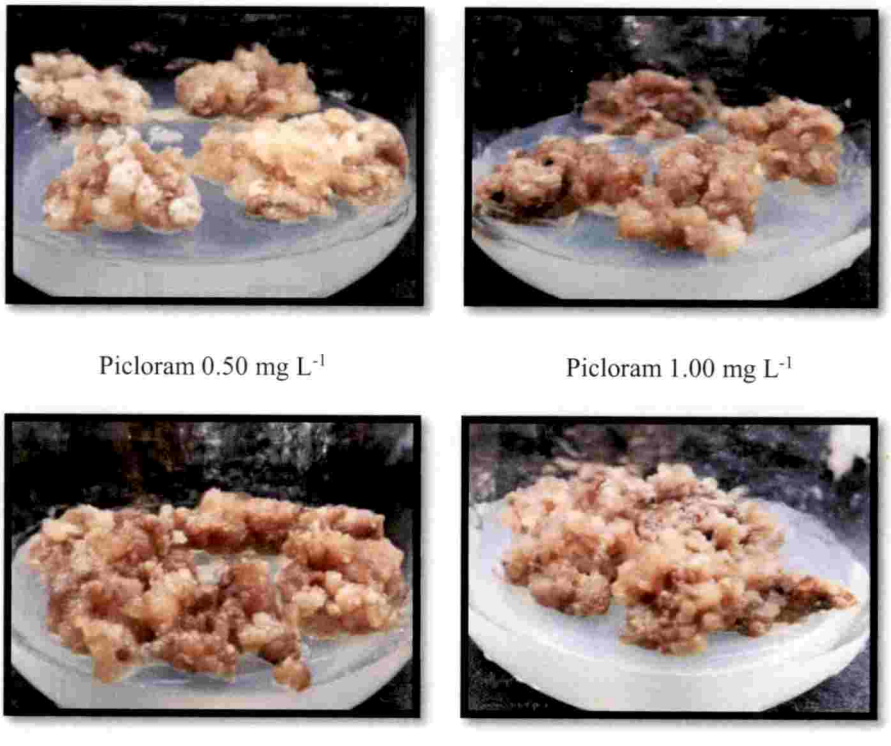
NAA 2.00 mg L^{-1} (15DAI)

NAA 2.00 mg L^{-1} (45DAI)

Plate 7: Effect of NAA in MS medium on callus induction and multiplication from intermodal explants

A) IBA 1.50 mg L^{-1} B) IBA 2.00 mg L^{-1} C) 2,4-D 2.00 mg L^{-1}

Plate 8: Effect of IBA and 2,4-D in MS medium on callus induction and multiplication from internodal explants, 45 days after inoculation



Picloram 0.50 mg L^{-1} Picloram 1.00 mg L^{-1}

Picloram1.50 mg L^{-1} Picloram 2.00 mg L^{-1}

Plate 9: Effect of picloram in MS medium on callus induction and multiplication from intermodal explants

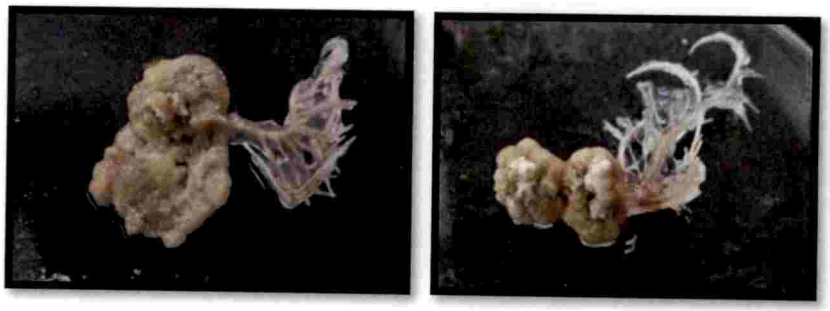
The eight treatments augmented with cytokinins alone, BA and Kn (C17 – C24) at various concentrations did not initiate callus.

In treatments involving combinations of BA and auxins alone, earliest (9.17 days) callusing was obtained in the treatments C29 followed by C31 (11.25days) and C32 (11.92 days). In C30 callus initiated in 12.75 days. All the treatments from C29 to C32 gave 100 per cent callus induction with a growth score of '3' (Plate 10). Callus formed in these treatments were hard and green. In BA - auxin combinations, the treatments supplemented with BA (2.00 mg L^{-1}) did not give callus initiation. The internodal explants just dried off.

Among the treatments supplemented with Kn - auxin combinations callus initiation was achieved only in C37 (MS + Kn 3.00 mg L^{-1} + NAA 1.00 mg L^{-1}) and C40 (MS + Kn 3.00 mg L^{-1} + 2,4-D 1.00 mg L^{-1}), in 16.78 days and 19.67 days respectively. Though both treatments gave 75 per cent callus induction, growth score was very low (1). Hard yellowish green callus that turned brown easily in these treatments (Plate 11).

Among the 44 treatments tried with internode as the explant, better callusing response with respect to days to callus initiation, callus induction percent and growth score, was obtained with treatments supplemented with picloram (C41, C42, C43, C44) followed by those supplemented with NAA (C3, C4) and BA-NAA combination (C29).

Leaf and internodal explants gave differential responses to varying levels of hormones (Fig 1). Leaf explants did not respond in NAA (C1 and C2) while internodal explants responded to all the four concentrations of NAA (C1, C2, C3, C4). In IAA supplemented medium (C5, C6, C7, C8), leaf explants showed root morphogenesis while the internodal explants did not respond at all. Leaf explants when inoculated to IBA supplemented media (C11 and C12), gave root morphogenesis without callus initiation but internodal explant gave callusing with



BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} BA 3.00 mg L^{-1} + IAA 1.00 mg L^{-1}

BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1} BA 3.00 mg L^{-1} + 2,4- D 1.00 mg L^{-1}

Plate 10: Effect of BA – auxin combinations in MS medium on callus induction and multiplication from internodal explants, 30 days after inoculation

Kn 3.00 mg L^{-1} + 2,4- D 1.00 mg L^{-1} Kn 3.00 mg L^{-1} + NAA 1.00 mg L^{-1}

Plate 11: Effect of Kn – auxin combinations in MS medium on callus induction and multiplication from internodal explants, 30 days after inoculation

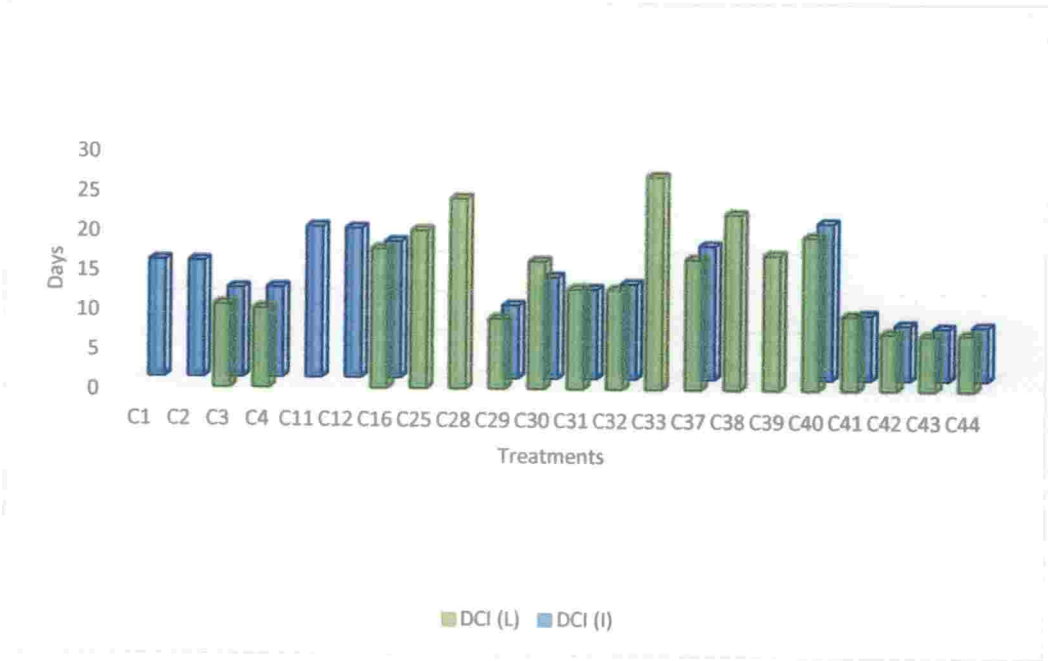


Fig. 1 Comparison of days to callus initiation from leaf and intermodal explants

- DCI (L) – Days to callus initiation from leaf explants
- DCI (I) – Days to callus initiation from internodal explants

root initials. In BA (2.00 mg L^{-1}) -auxin and Kn (2.00 mg L^{-1}) - auxin combination, leaf explant initiated callus in C25, C28, C33 and C36, but no response was obtained from internodal explant in any of these combinations. In treatments with Kn (3.00 mg L^{-1}) - auxin combinations, leaf explant responded in C37, C38, C39 and C40 but internodal explant responded only to C37 and C40. Thus, differential response was exhibited by the two explants with the various treatments tried.

4.1.2 Organogenesis

Seventy eight treatments with different hormonal combinations were tried to study organogenesis from callus in *C. frutescens*. Out of the seventy eight treatments, only two treatments, R37 (BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1}) and R61 (BA 5.00 mg L^{-1} + IAA 2.00 mg L^{-1}) gave shoots regeneration from callus. The calli obtained from C29 gave shoot regeneration. In R37 shoot initiation occurred in 41days and it attained a shoot length of 5.50 cm with 6 nodes by 65th day. The regenerated shoots had root initials (Plate 12 - A, B). The treatment R61 took 90 days to initiate shoot and recorded a shoot length of 1.50 cm with 2 nodes in 110 days (Plate 12 C). The results of the study are depicted in table 11.

4.1.3 Rooting of *in vitro* regenerated shoots

Sixteen treatments with varying concentrations of different auxins (NAA, IAA, IBA, 2,4-D) were tried to assess its effect on rooting. Out of the sixteen treatments, twelve treatments gave root initiation. Treatments Rt7 (MS + IAA 1.50 mg L^{-1}), Rt12 (MS + IBA 2.00 mg L^{-1}) and Rt15 (MS + 2,4-D 1.50 mg L^{-1}) recorded higher rooting percentage of 83.33 per cent. Rt10 (MS + IBA 1.00 mg L^{-1}) and Rt11 (MS + IBA 1.50 mg L^{-1}) recorded the least rooting per cent of 33.33. Rt2 (MS + NAA 1.00 mg L^{-1}), Rt3 (MS + NAA 1.50 mg L^{-1}), Rt4 (MS + NAA 2.00 mg L^{-1}) and Rt9 (MS + IBA 0.50 mg L^{-1}) did not respond to *in vitro* rooting. The results of the study are presented in table 12 and fig 2.

Table: 11 Effect of auxins and cytokinins in shoot regeneration from callus

Treatments	Cytokinins (mg L^{-1})		Auxins (mg L^{-1})			Days to shoot initiation	No. of shoot per callus inoculated	No. of nodes per shoot	Shoot length (cm)
	BA	Kn	NAA	IAA	IBA				
R1	1.00	-	-	-	-	-	-	-	-
R2	1.00	-	0.50	-	-	-	-	-	-
R3	1.00	-	1.00	-	-	-	-	-	-
R4	1.00	-	1.50	-	-	-	-	-	-
R5	1.00	-	2.00	-	-	-	-	-	-
R6	1.00	-	-	0.50	-	-	-	-	-
R7	1.00	-	-	1.00	-	-	-	-	-
R8	1.00	-	-	1.50	-	-	-	-	-
R9	1.00	-	-	2.00	-	-	-	-	-
R10	1.00	-	-	-	0.50	-	-	-	-
R11	1.00	-	-	-	1.00	-	-	-	-
R12	1.00	-	-	-	1.50	-	-	-	-
R13	1.00	-	-	-	2.00	-	-	-	-
R14	-	1.00	-	-	-	-	-	-	-
R15	-	1.00	0.50	-	-	-	-	-	-
R16	-	1.00	1.00	-	-	-	-	-	-
R17	-	1.00	1.50	-	-	-	-	-	-
R18	-	1.00	2.00	-	-	-	-	-	-
R19	-	1.00	-	0.50	-	-	-	-	-
R20	-	1.00	-	1.00	-	-	-	-	-
R21	-	1.00	-	1.50	-	-	-	-	-
R22	-	1.00	-	2.00		-	-	-	-
R23	-	1.00	-	-	0.50	-	-	-	-
R24	-	1.00	-	-	1.00	-	-	-	-
R25	-	1.00	-	-	1.50	-	-	-	-

Basal medium: MS medium

Table 11 continued....

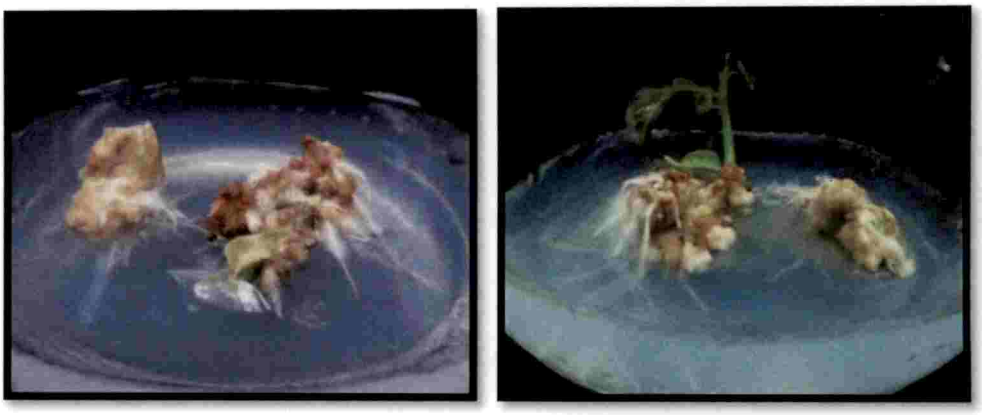
Treatments	Cytokinins (mg L^{-1})		Auxins (mg L^{-1})			Days to shoot initiation	No. of shoot per callus inoculated	No. of nodes per shoot	Shoot length (cm)
	BA	Kn	NAA	IAA	IBA				
R26	-	1.00	-	-	2.00	-	-	-	-
R27	3.00	-	-	-	-	-	-	-	-
R28	3.00	-	0.50	-	-	-	-	-	-
R29	3.00	-	1.00	-	-	-	-	-	-
R30	3.00	-	1.50	-	-	-	-	-	-
R31	3.00	-	2.00	-	-	-	-	-	-
R32	3.00	-	-	0.50	-	-	-	-	-
R33	3.00	-	-	1.00	-	-	-	-	-
R34	3.00	-	-	1.50	-	-	-	-	-
R35	3.00	-	-	2.00	-	-	-	-	-
R36	3.00	-	-	-	0.50	-	-	-	-
R37	3.00	-	-	-	1.00	41.00	1.00	6.00	5.50
R38	3.00	-	-	-	1.50	-	-	-	-
R39	3.00	-	-	-	2.00	-	-	-	-
R40	-	3.00	-	-	-	-	-	-	-
R41	-	3.00	0.50	-	-	-	-	-	-
R42	-	3.00	1.00	-	-	-	-	-	-
R43	-	3.00	1.50	-	-	-	-	-	-
R44	-	3.00	2.00	-	-	-	-	-	-
R45	-	3.00	-	0.50	-	-	-	-	-
R46	-	3.00	-	1.00	-	-	-	-	-
R47	-	3.00	-	1.50	-	-	-	-	-
R48	-	3.00	-	2.00	-	-	-	-	-
R49	-	3.00	-	-	0.50	-	-	-	-
R50	-	3.00	-	-	1.00	-	-	-	-

Basal medium: MS medium

Table 11 continued....

Treatments	Cytokinins (mg L^{-1})		Auxins (mg L^{-1})			Days to shoot initiation	No. of shoot per callus inoculated	No. of nodes per shoot	Shoot length (cm)
	BA	Kn	NAA	IAA	IBA				
R51	-	3.00	-	-	1.50	-	-	-	-
R52	-	3.00	-	-	2.00	-	-	-	-
R53	5.00	-	-	-	-	-	-	-	-
R54	5.00	-	0.50	-	-	-	-	-	-
R55	5.00	-	1.00	-	-	-	-	-	-
R56	5.00	-	1.50	-	-	-	-	-	-
R57	5.00	-	2.00	-	-	-	-	-	-
R58	5.00	-	-	0.50	-	-	-	-	-
R59	5.00	-	-	1.00	-	-	-	-	-
R60	5.00	-	-	1.50	-	-	-	-	-
R61	5.00	-	-	2.00	-	90.00	1.00	2.00	1.50
R62	5.00	-	-	-	0.50	-	-	-	-
R63	5.00	-	-	-	1.00	-	-	-	-
R64	5.00	-	-	-	1.50	-	-	-	-
R65	5.00	-	-	-	2.00	-	-	-	-
R66	-	5.00	-	-	-	-	-	-	-
R67	-	5.00	0.50	-	-	-	-	-	-
R68	-	5.00	1.00	-	-	-	-	-	-
R69	-	5.00	1.50	-	-	-	-	-	-
R70	-	5.00	2.00	-	-	-	-	-	-
R71	-	5.00	-	0.50	-	-	-	-	-
R72	-	5.00	-	1.00	-	-	-	-	-
R73	-	5.00	-	1.50	-	-	-	-	-
R74	-	5.00	-	2.00	-	-	-	-	-
R75	-	5.00	-	-	0.50	-	-	-	-
R76	-	5.00	-	-	1.00	-	-	-	-
R77	-	5.00	-	-	1.50	-	-	-	-
R78	-	5.00	-	-	2.00	-	-	-	-

Basal medium: MS medium



A) 45 days after inoculation

B) 65 days after inoculation

C) 95 days after inoculation

Plate 12: Shoot regeneration from callus in A &B) MS medium with BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1} C) MS medium with BA 5.00 mg L^{-1}+ IAA 2.00 mg L^{-1}

Table: 12 Effect of auxins on *in vitro* rooting of microshoots

Treatments	Auxins (mg L^{-1})				Days for root initiation	Rooting percentage	No. of roots per shoot	Root length (cm)
	NAA	IAA	IBA	2,4-D				
Rt1	0.50	-	-	-	16.00	50.00	2.00	2.18
Rt2	1.00	-	-	-	-	-	-	-
Rt3	1.50	-	-	-	-	-	-	-
Rt4	2.00	-	-	-	-	-	-	-
Rt5	-	0.50	-	-	15.67	50.00	2.00	3.74
Rt6	-	1.00	-	-	15.50	50.00	5.33	3.09
Rt7	-	1.50	-	-	12.83	83.33	5.67	4.53
Rt8	-	2.00	-	-	13.33	50.00	5.00	3.50
Rt9	-	-	0.50	-	-	-	-	-
Rt10	-	-	1.00	-	15.00	33.33	2.00	2.00
Rt11	-	-	1.50	-	16.00	33.33	2.00	2.50
Rt12	-	-	2.00	-	14.20	83.33	2.89	3.38
Rt13	-	-	-	0.50	19.67	66.67	4.17	3.03
Rt14	-	-	-	1.00	18.67	66.67	3.50	3.07
Rt15	-	-	-	1.50	18.50	83.33	4.50	3.44
Rt16	-	-	-	2.00	18.83	66.67	5.00	3.23
CD	-	-	-	-	1.3839	-	1.38	0.8482
f value	-	-	-	-	2.456	-	2.46	2.456

Treatments that gave rooting per cent less than 50 were omitted from statistical analysis.

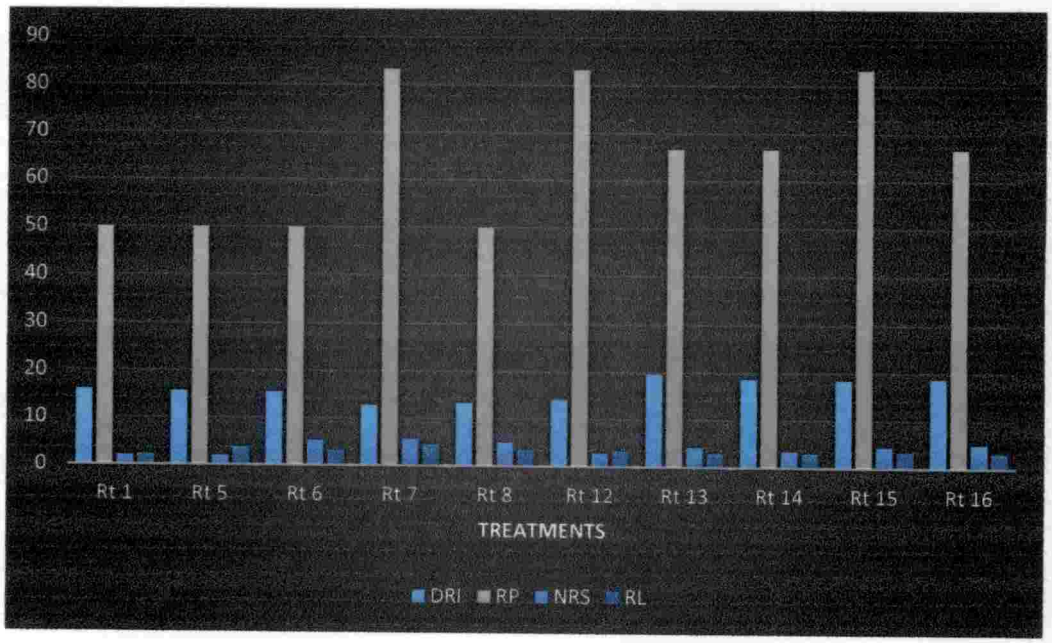


Fig. 2 Effect of auxins on *in vitro* rooting of microshoots

- DRI – Days to root initiation
- RP – Rooting percentage
- NRS – Number of roots per shoot
- RL – Root length

All the four different levels of IAA produced roots *in vitro*. Among the treatments with IAA, in Rt7, 83.33 per cent of the cultures initiated roots in 12 days and recorded maximum number of roots per shoot (5.67) with an average root length of 4.53 cm. IAA treatments produced sturdy roots. Whereas, other three treatments, Rt5 (MS + IAA 0.50 mg L^{-1}), Rt6 (MS +IAA 1.00 mg L^{-1}) and Rt8 (MS+IAA 2.00 mg L^{-1}) recorded only fifty per cent rooting and root initiation was observed in 15.67, 15.50 and 13.33 days respectively. The number of roots per shoot recorded in Rt5, Rt6 and Rt8 were 2, 5.33, and 5 with a shoot length of 3.74 cm, 3.09 cm and 3.50 cm, respectively. Though the treatments Rt6 and Rt8 gave only 50.00 percent rooting, root number per shoot in these two treatments were on par with Rt7.

Among the treatments supplemented with IBA, Rt12 (MS + IBA 2.00 mg L^{-1}), gave maximum rooting (83.33 per cent) with 2.89 roots per shoot and a shoot length of 3.38 cm. The root initiation was also the earliest (14.2 days) in this treatment, compared to other levels of IBA. Rt10 (MS + IBA 1.00 mg L^{-1}), and Rt11 (MS + IBA 1.50 mg L^{-1}) initiated roots in 15 and 16 days with a rooting per cent of 33.33. The number of roots per shoot in both treatments, Rt10 and Rt11 were 2.00 with a root length of 2.00 cm and 2.50 cm, respectively. Roots generated in IBA treatments were fragile, thin and short. Among the four levels of IBA 2.00 mg L^{-1} was the best in developing roots with respect to rooting per cent, days to root initiation, number of roots per shoot and root length. The treatment Rt9 (MS + IBA 0.50 mgL^{-1}) did not give any root initiation.

Out of the four concentrations of NAA, only one concentration, Rt1 (MS + NAA 0.50 mg L^{-1}) initiated roots in 16 days with a rooting per cent of 50.00. It produced 2 roots per shoot with a length of 2.18 cm. Treatments supplemented with higher concentration of NAA, Rt2 (MS + NAA 1.00 mg L^{-1}), Rt3 (MS + NAA 1.50 mg L^{-1}) and Rt4 (MS + NAA 2.00 mg L^{-1}) did not initiate roots.

The treatments supplemented with all the four concentrations of 2,4-D initiated roots. Among these, higher rooting per cent (83.33) was observed in Rt15 (MS + 2,4-D 1.50 mg L^{-1}). The other three levels of 2,4-D (Rt13, Rt14 and Rt16) recorded a rooting per cent of 66.67. The higher number of roots per shoot (5.00) was observed in Rt16 (MS + 2,4-D 2.00 mg L^{-1}) which was found to be on par with Rt15 (4.50). Rt15 recorded the higher root length of 3.44 cm followed by Rt16 (3.23 cm). The number of roots per shoot generated in Rt13 and Rt14 were 4.17 and 3.50 with a shoot length of 3.03cm and 3.07 cm, respectively. The roots produced in 2,4-D augmented media were short, thick and dense. The number of primary roots generated were less but with more number of secondary roots, compared to IAA supplemented treatments. The roots initiated when microshoots are inoculated to MS medium with various levels of auxins are depicted in Plate 13.

Among the 16 treatments, Rt7 (MS + IAA 1.50 mg L^{-1}) was the best with respect to days for root initiation, rooting per cent, number of roots per shoot and root length.

4.2 PHASE II STANDARDIZATION OF PROTOCOL FOR PROTOPLAST CULTURE

4.2.1 Protoplast isolation

Leaves excised from *in vitro* generated seedlings and callus multiplied on MS + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1}, were used for protoplast isolation studies. The two steps in protoplast isolation *viz.*, preplasmolysis and enzymatic isolation were combined for effective isolation of protoplast. The protoplast isolation solutions were prepared with varying concentrations of mannitol (0.40 M, 0.50 M, 0.60 M, 0.70M, 0.80M) and enzyme combinations of cellulase (1.00- 4.00 per cent) and pectinase (0.25 – 2.00 per cent) / Macerozyme R-10 (0.25 - 0.50 per cent) in CPW solution. The explants were incubated in these different protoplast isolation solutions for varying period ie, 2h, 4h, 6h, 14h and 16h with a shaking of 50 rpm at 27 °C in dark. After incubation, the protoplast yield and viability of the filtrate collected after

2,4-D 0.5 mgL^{-1} 2,4-D 1.0 mgL^{-1} 2,4-D 1.5 mgL^{-1} 2,4-D 2.0 mgL^{-1}

IAA0.5 mgL^{-1} IAA1.0 mgL^{-1} IAA1.5 mgL^{-1} IAA 2.0 mgL^{-1}

NAA0.5 mgL^{-1} IBA1.0 mgL^{-1} IBA1.5 mgL^{-1} IBA 2.0 mgL^{-1}

Plate 13: Effect of auxins in MS medium on *in vitro* rooting of microshoots

the removal of debris were determined by counting using haemocytometer. Protoplasts were stained using phenosafranine dye to assess the viability. Viable protoplasts remained green or colourless and dead protoplasts exhibited red/ pink colour. The viable and dead protoplasts isolated from leaf on treatment with digestion mixture containing mannitol 0.50 M + cellulase 2.00 per cent + macerozyme 0.50 per cent and incubated in dark for 6 h is given in Plate 14.

At 0.70 M mannitol, the protoplasts were found to shrink. So, the concentrations of mannitol, 0.70 M and beyond were exempted from the study. Also, when the enzyme combinations of cellulase and pectinase were tried, few or no protoplasts were formed. So, macerozyme, which is a combination of pectinase, hemicellulose and cellulase was used, instead of pectinase.

4.2.1.1 Protoplast isolation from leaf

Among the sixty protoplast isolation treatments, the best response of leaf with respect to protoplast yield and viability was obtained in treatment DM_{28} (Cellulase 2.00 per cent + macerozyme 0.50 per cent + mannitol 0.50 M in CPW solution and incubation in dark for 6 h). The protoplast yield was 124 $x10^5$ with 95.16 per cent viability.

It was observed in the study that at mannitol 0.40 M concentration, all the different enzyme combinations gave maximum protoplast yield at 6 h of incubation (Table 13). At all levels of mannitol concentration, different enzyme combinations gave higher yield at 6 h of incubation. At 14 h and 16 h of incubation protoplast yield decreased substantially in all protoplast isolation treatments. It was also noted that at mannitol 0.60 M concentration, protoplast yield decreased significantly compared to lower levels of mannitol concentrations. At mannitol 0.60 M concentration, higher concentration of enzyme mixture combinations (Cellulase 4.00 per cent +

Table 13: Effect of different osmotica, enzyme combinations and periods of exposure on protoplast yield and viability from *in vitro* derived leaves

Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per g tissue)	Protoplast viability (%)
DM_1	0.4 M	Cellulase 1% + macerozyme 0.25%	2 h	9.33×10^5	100.00
DM_2			4 h	70.66×10^5	93.40
DM_3			6 h	79.33×10^5	92.44
DM_4			14 h	31.33×10^5	72.34
DM_5			16 h	29.33×10^5	77.27
DM_6		Cellulase 2% + macerozyme 0.50%	2 h	12.66×10^5	94.74
DM_7			4 h	39.33×10^5	92.86
DM_8			6 h	66.66×10^5	95.00
DM_9			14 h	46.66×10^5	89.83
DM_{10}			16 h	42.00×10^5	84.13
DM_{11}		Cellulase 4% + macerozyme 0.25%	2 h	58.66×10^5	94.32
DM_{12}			4 h	59.33×10^5	93.26
DM_{13}			6 h	78.66×10^5	95.76
DM_{14}			14 h	72.00×10^5	89.81
DM_{15}			16 h	14.00×10^5	77.27
DM_{16}		Cellulase 4% + macerozyme 0.50%	2 h	5.33×10^5	90.00
DM_{17}			4 h	70.00×10^5	73.75
DM_{18}			6 h	67.33×10^5	83.17
DM_{19}			14 h	53.33×10^5	89.52
DM_{20}			16 h	4.00×10^5	33.33
DM_{21}	0.5 M	Cellulase 1% + macerozyme 0.25%	2 h	52.00×10^5	92.31
DM_{22}			4 h	50.66×10^5	84.21
DM_{23}			6 h	102.66×10^5	87.01
DM_{24}			14 h	74.66×10^5	77.68
DM_{25}			16 h	53.33×10^5	67.50

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking

Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.

Table 13 continued....

Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per g tissue)	Protoplast viability (%)
DM_{26}	0.5 M	Cellulase 2% + macerozyme 0.50%	2 h	51.33×10^5	93.51
DM_{27}			4 h	53.33×10^5	93.75
DM_{28}			6 h	124.00×10^5	95.16
DM_{29}			14 h	54.66×10^5	84.15
DM_{30}			16 h	50.00×10^5	76.00
DM_{31}		Cellulase 4% + macerozyme 0.25%	2 h	42.00×10^5	74.60
DM_{32}			4 h	60.00×10^5	86.76
DM_{33}			6 h	90.66×10^5	82.20
DM_{34}			14 h	54.66×10^5	70.73
DM_{35}			16 h	20.66×10^5	64.52
DM_{36}		Cellulase 4% + macerozyme 0.50%	2 h	36.00×10^5	79.71
DM_{37}			4 h	46.00×10^5	74.07
DM_{38}			6 h	38.00×10^5	66.67
DM_{39}			14 h	26.66×10^5	58.06
DM_{40}			16 h	20.66×10^5	50.00
DM_{41}	0.6 M	Cellulase 1% + macerozyme 0.25%	2 h	38.00×10^5	96.49
DM_{42}			4 h	31.33×10^5	82.98
DM_{43}			6 h	27.33×10^5	75.61
DM_{44}			14 h	21.33×10^5	62.50
DM_{45}			16 h	16.66×10^5	62.50
DM_{46}		Cellulase 2% + macerozyme 0.50%	2 h	22.00×10^5	78.79
DM_{47}			4 h	9.33×10^5	76.47
DM_{48}			6 h	14.66×10^5	63.64
DM_{49}			14 h	9.00×10^5	42.86
DM_{50}			16 h	6.00×10^5	42.86
DM_{51}		Cellulase 4% + macerozyme 0.25%	2 h	15.33×10^5	81.82
DM_{52}			4 h	6.00×10^5	80.00
DM_{53}			6 h	2.00×10^5	79.41
DM_{54}			14 h	-	-
DM_{55}			16 h	-	-

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking

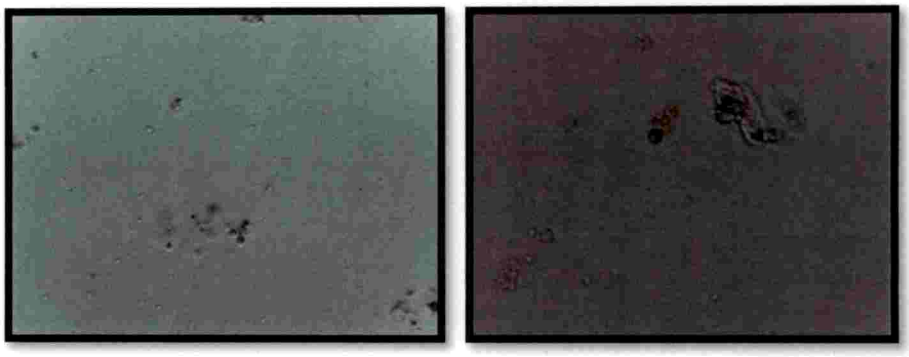
Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.

Table 13 continued....

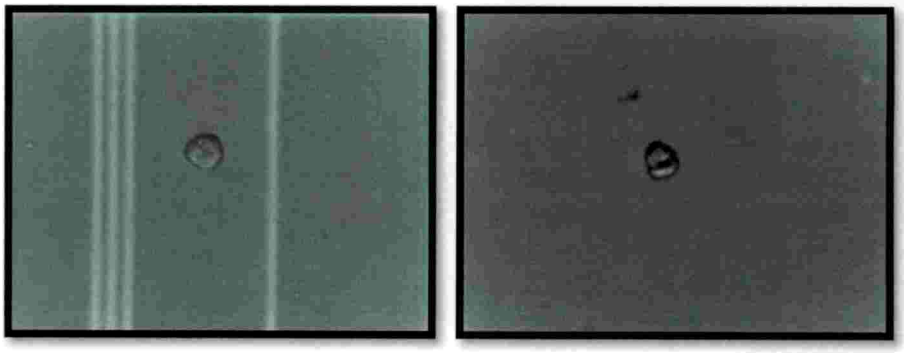
Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per g tissue)	Protoplast viability (%)
DM_{56}		Cellulase 4% + macerozyme 0.50%	2 h	11.33×10^5	63.64
DM_{57}			4 h	2.00×10^5	60.00
DM_{58}			6 h	2.00×10^5	62.50
DM_{59}			14 h	-	-
DM_{60}			16 h	-	-
CD	-	-	-	16.22	-
f value	-	-	-	1.48	-

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking

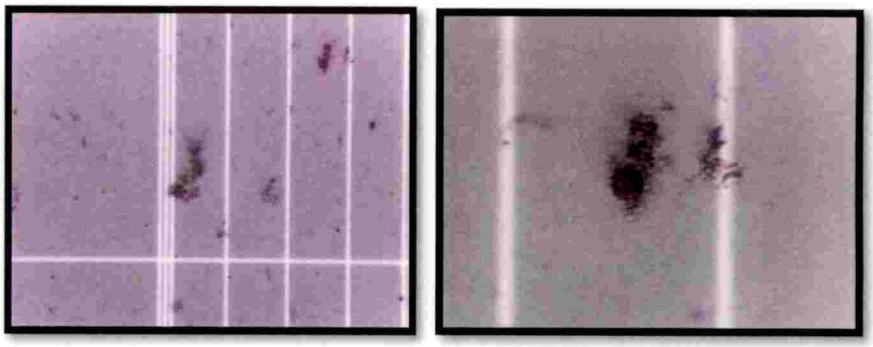
Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.



Viable protoplast (400 x)



Viable protoplast (1000 x)



Dead protoplast (400 x)

Dead protoplast (1000 x)

Plate 14: Protoplast isolated from leaf on treatment with digestion mixture containing mannitol 0.50 M + cellulase 2.00 per cent + macerozyme 0.50 per cent and incubated in dark for 6 h

macerozyme 0.25, 0.50 percent), did not yield protoplast at 14 h and 16 h of incubation.

4.2.1.2 Protoplast isolation from callus

Among the 60 protoplast isolation treatments, protoplast yield (36.00 $x10^5$) from callus was the highest in treatment DM_{47} (Cellulase 2.00 per cent + macerozyme 0.50 per cent + mannitol 0.60 M in CPW solution and incubation in dark for 4 h). This is on par with D_{48} (Cellulase 2.00 per cent + macerozyme 0.50 per cent + mannitol 0.60 M in CPW solution and incubation in dark for 6 h) and D_{51} (Cellulase 4.00 per cent + macerozyme 0.25 per cent + mannitol 0.60 M in CPW solution and incubation in dark for 2 h). The protoplast viability recorded in treatments DM_{47}, DM_{48} and DM_{51} were 85.30, 85.21 and 79.00 per cent, respectively (Table 14).

The protoplast yield and viability were observed to be better with respect to the leaf explant compared to callus.

4.2.2 Protoplast washing and purification

The protoplast isolated in the best protoplast isolation treatment was subjected to centrifugation at 700 rpm, 900 rpm and 2000 rpm for pelleting the protoplast. Centrifugation at 700 rpm and 900 rpm did not form pellet but with 2000 rpm. The pelleted protoplast dissolved in CPW solution was layered on floatation medium with varying concentration of sucrose (15, 21 and 25 per cent) and further centrifuged for purification. The viable protoplast formed a layer at interphase between CPW solution and floatation media. It was observed that floatation medium with 21 per cent sucrose gave higher protoplast yield and viability in both leaf as well as callus. At 21 per cent, leaf recorded a protoplast yield of $30x10^5$ protoplasts per g with 90.91 per cent viability while callus recorded a lower yield of 10 x 10^5 protoplasts per g with 100 per cent viability (Table 15).

Table 14: Effect of different osmotica, enzyme combinations and periods of exposure on protoplast yield and viability from calli

Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per g calli)	Protoplast viability (%)
DM_1	0.4 M	Cellulase 1% + macerozyme 0.25%	2 h	-	-
DM_2			4 h	5.33×10^5	100.00
DM_3			6 h	7.33×10^5	100.00
DM_4			14 h	4.67×10^5	92.30
DM_5			16 h	4.00×10^5	91.25
DM_6		Cellulase 2%+ macerozyme 0.50%	2 h	5.33×10^5	100.00
DM_7			4 h	6.00×10^5	97.30
DM_8			6 h	8.67×10^5	97.00
DM_9			14 h	10.67×10^5	96.56
DM_{10}			16 h	8.33×10^5	90.00
DM_{11}		Cellulase 4% + macerozyme 0.25%	2 h	5.33×10^5	98.00
DM_{12}			4 h	8.00×10^5	90.01
DM_{13}			6 h	13.33×10^5	85.23
DM_{14}			14 h	9.33×10^5	85.45
DM_{15}			16 h	4.67×10^5	83.05
DM_{16}		Cellulase 4% + macerozyme 0.50%	2 h	12.00×10^5	85.00
DM_{17}			4 h	10.00×10^5	81.45
DM_{18}			6 h	5.30×10^5	74.00
DM_{19}			14 h	4.00×10^5	72.36
DM_{20}			16 h	2.67×10^5	72.65
DM_{21}	0.5 M	Cellulase 1% + macerozyme 0.25%	2 h	13.33×10^5	93.00
DM_{22}			4 h	13.33×10^5	94.50
DM_{23}			6 h	13.33×10^5	90.10
DM_{24}			14 h	14.00×10^5	82.50
DM_{25}			16 h	8.67×10^5	80.29

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking.

Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.

Table 14 continued....

Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per g calli)	Protoplast viability (%)
DM_{26}	0.5 M	Cellulase 2% + macerozyme 0.50%	2 h	14.00x 10^5	100.00
DM_{27}			4 h	18.67x 10^5	94.00
DM_{28}			6 h	26.67x 10^5	94.00
DM_{29}			14 h	26.67x 10^5	81.45
DM_{30}			16 h	18.67x 10^5	80.00
DM_{31}		Cellulase 4% + macerozyme 0.25%	2 h	16.00x 10^5	85.01
DM_{32}			4 h	23.33x 10^5	82.36
DM_{33}			6 h	20.00x 10^5	79.23
DM_{34}			14 h	16.00x 10^5	80.09
DM_{35}			16 h	10.00x 10^5	72.36
DM_{36}		Cellulase 4% + macerozyme 0.50%	2 h	12.00x 10^5	85.00
DM_{37}			4 h	24.67x 10^5	82.36
DM_{38}			6 h	17.33x 10^5	78.81
DM_{39}			14 h	17.33x 10^5	73.25
DM_{40}			16 h	10.67x 10^5	73.25
DM_{41}	0.6 M	Cellulase 1%+ macerozyme 0.25%	2 h	15.33x 10^5	89.30
DM_{42}			4 h	22.00x 10^5	89.00
DM_{43}			6 h	23.37x 10^5	89.21
DM_{44}			14 h	23.33x 10^5	83.04
DM_{45}			16 h	7.33x 10^5	79.60
DM_{46}		Cellulase 2%+ macerozyme 0.50%	2 h	14.00x 10^5	89.35
DM_{47}			4 h	36.00x 10^5	85.30
DM_{48}			6 h	29.33x 10^5	85.21
DM_{49}			14 h	22.00x 10^5	83.50
DM_{50}			16 h	-	-
DM_{51}		Cellulase 4% + macerozyme 0.25%	2 h	35.33x 10^5	79.00
DM_{52}			4 h	20.67x 10^5	80.56
DM_{53}			6 h	13.33x 10^5	75.00
DM_{54}			14 h	14.00 x 10^5	73.25
DM_{55}			16 h	-	-

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking.

Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.

Table 14 continued....

Treatment	Concentration of mannitol	Enzyme combination	Time period	Protoplast yield (per gm calli)	Protoplast viability (%)
DM_{56}		Cellulase 4% + macerozyme 0.50%	2 h	-	-
DM_{57}			4 h	12.00×10^5	83.01
DM_{58}			6 h	-	-
DM_{59}			14 h	-	-
DM_{60}			16 h	-	-
CD	-	-	-	8.66	-
f value	-	-	-	1.47	-

Temperature – 27°C, p^H – 5.8, Incubation in dark at 50 rpm shaking.

Treatments that did not yield protoplast in all the three replications were eliminated from statistical analysis.

Table 15: Effect of floatation medium containing different levels of sucrose on protoplast yield and viability

Sl No	Concentration of FM	Protoplast yield (per gm leaf/calli)		Protoplast viability (%)	
		Leaves	Callus	Leaves	Callus
FM 1	Sucrose 15 %	22 x 10^5	2.0 x 10^5	90.91	100.00
FM 2	Sucrose 21 %	30x 10^5	10 x 10^5	90.91	100.00
FM 3	Sucrose 25 %	10 x10^5	4.0 x 10^5	88.80	100.00

FM – Floatation medium

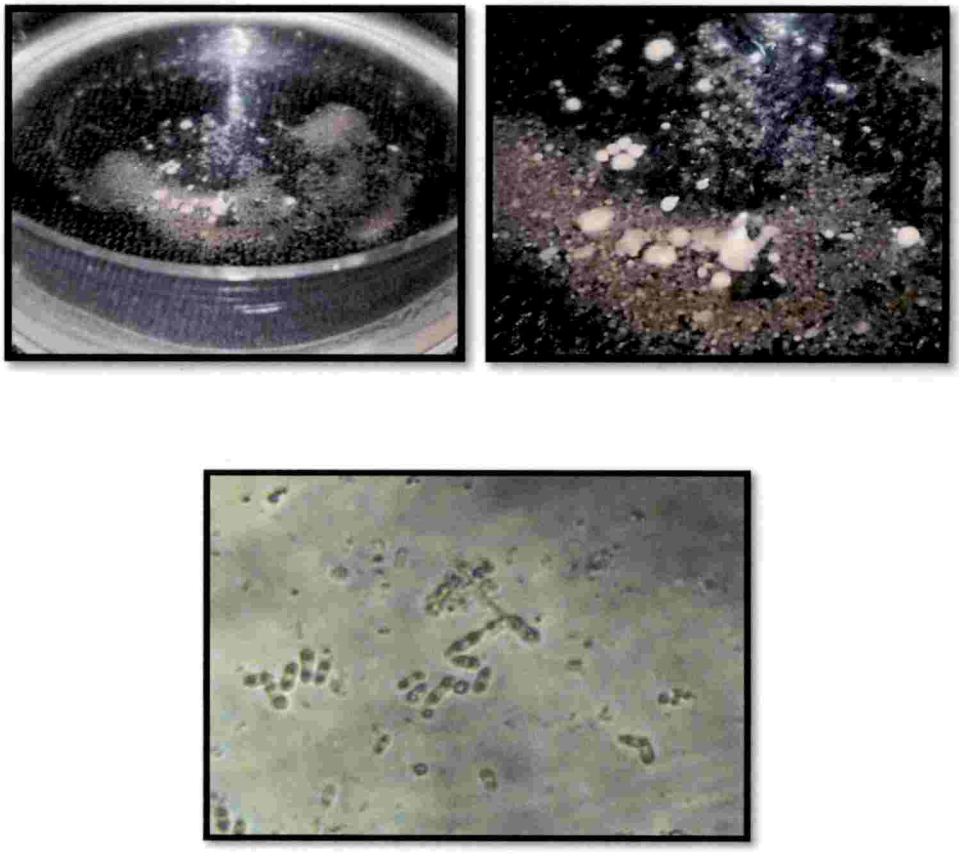
4.2.3 Protoplast culture

The purified protoplasts at different plating densities ($2x10^5$, $5x10^5$, $10x10^5$) were cultured on liquid MS culture medium (CMI) supplemented with varying concentrations of 2,4- D (0.50 - 6.00 mg L^{-1}) and mannitol (0.40 - 0.50 M), in petri plates. The microcalli initiation from leaf as well as callus was observed in treatment PCM_5 (liquid MS culture medium + 2,4-D 0.50 mg L^{-1} + mannitol 0.50 M) within 45 days. After a week, CMII solution was added and further development of microcalli was observed in 60 days for callus derived protoplast and 70 days in leaf derived protoplast (Plate 15). The results of the study are presented in table 16.

Table 16: Days to visible microcalli colony formation from protoplasts

Treatments	Composition of CM I	Plating density	Days to microcalli initiation	Days to visible microcalli colony formation	
				P_L	P_C
PCM 1	L MS + mannitol 0.40 M + 2,4- D 0.50 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 2	L MS + mannitol 0.40 M + 2,4- D 2.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 3	L MS + mannitol 0.40 M + 2,4- D 4.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 4	L MS + mannitol 0.40 M + 2,4- D 6.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 5	L MS + mannitol 0.50 M + 2,4- D 0.50 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	45	70	60
PCM 6	L MS + mannitol 0.50 M + 2,4- D 2.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 7	L MS + mannitol 0.50 M + 2,4- D 4.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-
PCM 8	L MS + mannitol 0.50 M + 2,4- D 6.00 mgL^{-1}	2×10^5	-	-	-
		5×10^5	-	-	-
		10×10^5	-	-	-

CM- Culture medium, L MS – Liquid MS. After microcalli initiation in CM I, CMII was added for further development of visible colonies of microcalli.



400 x

Plate 15: Microcalli formed after 70 days in culture medium containing liquid MS + mannitol 0.50 M + 2,4-D 0.50 mg L^{-1}

Discussion

5. DISCUSSION

The present study, "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L." was carried out at Department of Plant Biotechnology, College of Agriculture, Vellayani. The results obtained in the study are discussed in this chapter.

5.1 ESTABLISHMENT OF CALLUS CULTURE AND ORGANOGENESIS

5.1.1 Establishment of callus culture

The combinations of endogenous hormones produced in the tissue and exogenous hormones supplemented in the medium defines the tissue response (Duclercq *et al.*, 2011). *In vitro* callus induction and regeneration could be obtained by using auxins alone or in combination with cytokinin.

In the present study, effect of different plant growth hormones on callus induction and multiplication was studied. Among the various auxins tried, picloram and NAA gave maximum callus induction and multiplication. Rao and Sangapure (2014) also observed effective callusing in NAA 2.00 mg L^{-1} supplemented MS medium in *C. annuum*. In the study, NAA produced yellowish green callus with profuse multiplication, which is in confirmation with the observation of Ray *et al.* (2011) in *Solanum melongena*, where NAA produced greenish, fast-growing callus. In contrast to this, Jadhav *et al.* (2014) reported that NAA did not induce callus formation in *S. melongena*.

In this study, picloram (0.50 mg L^{-1}– 2.00 mg L^{-1}) produced soft white profuse calli (100.00 per cent) both from leaf (6.83 to 9.25 days) and internodal explants (6.58 to 8.17 days). The effect of picloram in callus induction was also reported in other crop sp. like banana (Houllou-kido *et al.*, 2005; Smitha *et al.* 2011;

Lekshmi, 2016), barley (Sener *et al.*, 2016) and *Verbena bipinnatifida* (Genady, 2017). Immature male inflorescence of banana developed callus (80.00 per cent) when inoculated to MS + picloram after two months of inoculation (Houllou-kido *et al.*, 2005). Smitha *et al.* (2011) reported that banana leaf sheath developed brown spongy callus with yellow globular structures with lower concentrations of picloram in MS medium and brown compact callus with white globular structures with higher concentrations of picloram. Lekshmi (2016) obtained callus induction (60 per cent) from immature male inflorescence of banana (*Musa sp.*) in 45 days in MS + picloram (3.00 mg L^{-1} – 10.00 mg L^{-1}). Immature inflorescence of barley (*Hordeum vulgare*) gave highest callus induction (31.3 per cent) in MS + picloram 7.5 mg L^{-1} (Sener *et al.*, 2016). According to Genady (2017), picloram 2.00 mg L^{-1} is the best for callus induction in *Verbena bipinnatifida*.

In this study, 100 per cent callus induction was also observed in MS medium supplemented with BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} and callus initiated in about 9.17 days. This is in line with the observation by Raj *et al.* (2015) in *C. chinense*, where successful induction of callus from stem segments of *in vitro* raised plants was obtained in MS + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} after two weeks of culture and 91.01 per cent cultures showed callus development. NAA – BAP combination were effective in inducing callus in other solanceous crop like brinjal, potato etc. Ray *et al.* (2011) also observed the highest per cent (48.66) of callus initiation from stem segments of brinjal in MS + BAP 2.00 mg L^{-1} + NAA 0.50 mg L^{-1} within 8.20 days. According to Kumlay and Ercisli (2015), MS medium with BAP 3.00 mg L^{-1} + NAA 2.00 mg L^{-1} induced callusing in a minimum of 8.33 days in potato. Nasrin *et al.* (2003) observed highest callus development from leaf and internodal explants of potato in MS medium with NAA – BAP combination.

In the present study, 2,4-D initiated hard callus both from leaf and internodal explants. But, the callus induction was only 50 per cent and 75 per cent in leaf and

internode, respectively. It also took more days for callus initiation compared to other treatments. Ray *et al.* (2011) reported that 2,4-D induced early callus production from the petiole in brinjal. It was observed in the study that MS + 2,4-D 1.00 mg L^{-1} + Kn 3.00 mg L^{-1} gave 100 per cent callus induction with a growth score of '2' in leaf explant and 75.00 per cent callus induction with a growth score of '1' in internodal explants. Mangang (2014) reported profuse callusing in MS medium + 2,4-D 2.00 mg L^{-1} + Kn 0.50 mg L^{-1}, from fresh immature green pods of *C. chinense*.

In the study, both leaf and internodal segments, when inoculated on to MS + BA 3.00 mg L^{-1} + 2,4-D 1.00 mg L^{-1}, gave 100 per cent callus initiation. Leaf explants initiated callus in 12.42 days with a growth score of '2'. Whereas, internodal explants initiated callus in 11.92 days with a growth score of '3'. Callus formed in these treatments were hard and green. Santos *et al.* (2017) reported higher callus induction per cent in BA- 2,4-D combination in MS medium from leaf and internodal explants of *C. annuum*. But calli formed was white and friable. Laboney *et al.* (2013) recorded the best callus induction of 95.00 per cent in 10.00 days, when leaf explants were inoculated on to MS medium with 2,4-D 2.00 mg L^{-1} in potato cv. Granola.

Leaf and internodal explants failed to initiate callus in MS medium with different concentration (0.50 mg L^{-1} – 2.00 mg L^{-1}) of IAA. The leaf explants initiated roots while internodal segments did not show any morphogenetic response. In contrast to our finding, Luis (2005) reported that shoot apices, internodes, anthers, and petioles when exposed to IAA produced profuse calli in *C. baccatum*.

5.1.2. Organogenesis

Chilli is recalcitrant to *in vitro* regeneration (Kothari *et al.*, 2010). The severe recalcitrant morphogenic nature, formation of rosette shoots and ill-defined shoot buds and genotypic dependent tissue culture responses have hindered progress in

tissue culture advancements and other biotechnological interventions in chilli (Reddy *et al.*, 2014). According to Skoog and Miller (1957), *in vitro* callus induction and regeneration could be obtained by using auxins alone or in combination with cytokinin. BAP is beneficial for inducing callus, followed by organogenesis in potato (Nasrin *et al.*, 2003). MS medium containing cytokinin - auxin combination lowered the days for shoot induction and enhanced number of shoots, shoot growth, number of nodes and leaves per plantlet in potato (Kumlay and Ercisli, 2015).

Seventy eight treatments with different hormonal combinations were tried to study organogenesis from callus in *C. frutescens*. Calli derived from the three treatments that gave better callusing were inoculated to different regeneration media. But calli derived from the treatment, C 29 (MS+ BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1}) only, gave shoot regeneration. Calli (leaf and internodal derived) obtained in MS medium containing the combination of NAA and BAP gave the highest shoot regeneration in potato (Nasrin *et al.*, 2003). Kumlay and Ercisli (2015) claimed that dark green and compact calli were capable of regenerating shoots in potato. Callus obtained from MS+ BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} initiated shoots in *C. chinense* (Raj *et al.*, 2015).

Callus mediated shoot regeneration was obtained in hormonal combinations with higher concentration of cytokinin (BA) and low concentrations of auxins (IBA and IAA). In this study, shoot regeneration with root initials was obtained from callus in MS + BA 3.00 mg L^{-1}+ IBA 1.00 mg L^{-1} and without shoot initials in MS + BA 5.00 mg L^{-1}+ IAA 2.00 mg L^{-1}. In consensus with the finding, Jan *et al.* (2015) reported maximum indirect regeneration in BAP- IAA augmented MS medium in hybrid tomato. Raj *et al.* (2015) obtained callus mediated plant regeneration of *C. chinense* in MS medium augmented with BA 5.00 mg L^{-1} and IAA 0.50 mg L^{-1}.

Regeneration media which consists of Kn and auxins did not respond to shoot regeneration. In contrast to this, Shirin *et al.* (2007) reported that Kn - NAA combination is more effective for shoot regeneration from internode derived calli in potato. According to Rakshit *et al.* (2008), the regeneration efficiency of the plant decreased in MS medium supplied with kinetin (2.00 mg L^{-1}) and NAA (0.50 mg L^{-1}) in brinjal. Kumlay and Ercisli (2015) obtained 72.50 per cent and 67.50 per cent of shoot regeneration in MS + BAP 2.00 mg L^{-1} + NAA 0.25 mg L^{-1} and MS + Kn 2.00 mg L^{-1} + NAA 0.25 mg L^{-1} respectively.

Shoot regeneration requires specific levels of the different growth hormones *viz.*, auxin and cytokinin. Different plant species exhibit variable responses to the exogeneous growth hormones. The response of a plant species to an exogeneous growth hormone may therefore, depend on the endogeneous level of that growth hormone in the species.

5.1.3 *In vitro* rooting of micro shoots

In this study, we tried sixteen treatments with varying concentrations of different auxins (NAA, IAA, IBA, 2,4-D) to assess its effect on rooting. *In vitro* rooting was obtained in low concentration of NAA (0.50 mg L^{-1}), all concentrations of IAA (0.50 mg L^{-1} – 2.00 mg L^{-1}), all concentrations of 2,4-D (0.50 mg L^{-1}- 2.00 mg L^{-1}) and higher concentrations of IBA (1.00 mg L^{-1}- 2.00mg L^{-1}).

NAA at lower level (0.50 mg L^{-1}) initiated small roots whereas at higher levels, it did not initiate shoots.

All the levels of IAA in MS medium induced roots in microshoots. The earliest rooting (12.83 days) was observed in MS + IAA 1.50 mg L^{-1}. This treatment recorded the highest number of roots per shoot (5.67), maximum root length (4.53

cm) and maximum rooting per cent of 83.33. Alizah and Zamri (2016) obtained root initiation from microshoots of *C. annuum* when inoculated on to MS + IAA0.50 mg L^{-1} after 2 weeks of culture.

In this study, MS medium containing IBA 2.00 mg L^{-1} gave root initiation in 14.20 days with 2.89 roots per shoot, root length of 3.38 cm and a rooting percentage of 83.33 per cent. Jadhav *et al.* (2014) recorded *in vitro* rooting of microshoots of brinjal, on MS supplemented with IBA 1.00 mg L^{-1} in 14.60 days with 90.00 per cent rooting. In our study, MS supplemented with IBA 1.00 mg L^{-1}gave root initiation in 15.00 days but a lower rooting percentage of 33.33 per cent.

Also, *in vitro* roots were initiated in MS medium supplemented with 2,4-D (0.50 mg L^{-1} – 2.00 mg L^{-1}). Hence, this study demonstrated that 2,4-D can also be used for inducing roots *in vitro* in *C. frutescens*. Short, sturdy and stout roots with more number of secondary roots were produced in 2,4-D supplemented MS medium. 2,4-D is usually used for inducing callus. Islam *et al.* (2005) reported that 2,4-D did not induce roots but was very effective in inducing rapid callus proliferation in *Cicer arietinum*. However, in consensus with the findings of the present study, Kumar *et al.* (2011) reported root induction occurred in 2,4-D at the concentration of 2.00 mg L^{-1}, with good root biomass in *Solanum trilobatum*.

5.2 STANDARDISATION OF PROTOCOL FOR PROTOPLAST CULTURE

The protoplast culture in this study involved enzymatic isolation of protoplast, its washing, purification and subsequent culture resulting in microcalli development.

The protoplast isolation was done combining preplasmolysis (using the osmoticum, mannitol) and enzymatic incubation of leaf tissues and calli, leading to the digestion of cell wall and, release of protoplast.

Leaves excised from *in vitro* generated seedlings and callus multiplied on MS + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1}, was used for protoplast isolation studies. Five different levels of mannitol (0.40 M, 0.50 M, 0.60 M, 0.70M, 0.80M), four different enzyme combinations of cellulase (1.00- 4.00 per cent) and pectinase (0.25 – 2.00 per cent) / Macerozyme R-10 (0.25 - 0.50 per cent) and five different time periods (2h, 4h, 6h,14h and 16h) of incubation with a shaking of 50 rpm at 27°C in dark were assessed to optimize the release of protoplast from *in vitro* germinated leaves and callus in *C. frutescens*.

The type and concentration of enzymes were important for isolation of protoplasts (Shao *et al.*, 2008). In this study, initially the enzyme mixtures of cellulase and pectinase were used but no protoplast could be obtained, which may be due to the resistance of cell wall to this enzyme combination. However, pectinase when replaced with macerozyme, formation of individual protoplasts was observed. Macerozyme 1.00 per cent encompasses 0.50 units mg^{-1} pectinase, 0.25 units mg^{-1} hemicellulase and 0.10 units mg^{-1} cellulase. Saxena *et al.* (1981) described the isolation of protoplasts from axenic shoot cultures of *C. annuum* cv. California wonder by using macerozyme 0.40 per cent and cellulase 2.00 per cent. According to Ayeleso (2015), enzymes mixtures of cellulases, hemicellulases, and pectinases are required to break cell wall towards protoplast isolation.

Osmotic values of the environment into which protoplasts are released are critically important. Different plant species, tissues and organs require different concentration of D-mannitol for the isolation of protoplasts (Kamnoon *et al.*, 2001; Duquenne *et al.*, 2007). Generally, the concentration of D-mannitol is 0.23 to 0.90 M; however, protoplasts are more stable in a slightly hypotonic environment when compared to isotonic environment, as a higher osmotic potential prevents the protoplasts bursting, but can inhibit their division (Bhojwani, 1996).

When mannitol at a concentration of 0.40 M and 0.50 M were used for protoplast isolation, protoplasts obtained from leaves were round and at mannitol 0.60 M, it started to shrink and protoplast yield declined. At mannitol 0.70 M it exhibited complete shrinkage of leaf derived protoplasts. But protoplast release from calli started to decline beyond mannitol 0.60 M. Mukhtar *et al.* (2012) reported that protoplast isolation solution containing mannitol 0.40 M was found to be effective for releasing and maintaining viable protoplasts from young leaves of *Dalbergia sissoo.*

When leaf explants were incubated in various enzyme combinations (DM_1 to DM_{15}, DM_{21} to DM_{35}) containing mannitol at 0.40 M and 0.50 M, protoplast yield showed an increasing trend with incubation period up to 6 h followed by a decreasing trend at longer incubation periods. But in enzyme combinations (DM_{16} to DM_{20}, DM_{36} to DM_{40}) containing mannitol 0.40 M and 0.50 M and higher concentration of cellulase 4.00 per cent and macerozyme 0.50 per cent, protoplast yield increased up to 4 h and then decreased with longer periods of incubation. Sura *et al.* (2015) obtained higher protoplast yield (5.8×10^5 protoplasts/ g) from mesophyll tissue of *C. annuum,* when incubated in enzyme mixture of cellulase 1.00 per cent and macerozyme 0.20 per cent. They reported that the number of protoplast released increased upto 4½ h of incubation and beyond which the protoplast yield gradually decreased and further resulted in complete shrinkage of protoplasts at 8-9 h of incubation. However, Huang *et al.* (2013) reported higher protoplast yield ($6 - 7 \times 10^6$ protoplast/g fresh weight) and viability (90 per cent)from leaves of *Cucumis sativus* by incubating in an enzyme solution containing cellulase 1.50 per cent, macerozyme 0.40 per cent, mannitol 0.40 M, for a time period of 8 h. These findings indicate that at higher enzyme concentration, the period of incubation required for release of protoplast may be less.

Length of incubation of tissues in enzyme solution also greatly influences the release of protoplasts from plant tissues. When plant tissues are incubated for a longer

time in lytic enzymes, it increases the chances of lysis of already formed protoplasts thereby decreasing the total protoplast yield (Ayeleso, 2015). In this study, it was found that an incubation for 6 h in dark, yielded maximum number of protoplasts from leaf explants. Protoplast yield from calli was maximum at 4h and 6h of incubation. In both, leaf as well as calli, a declining trend was observed in protoplast yield with longer periods of incubation. However, Diaz *et al.* (1988) reported protoplast isolation from leaf of *C. annuum* after 14 h of incubation in dark, when treated with an enzyme mixture of cellulase 1.00 per cent and macerozyme 0.25 per cent. Yoo *et al.* (2007) reported that prolonged incubation of leaves for 16-18 h is stressful for protoplasts but might be important for the dedifferentiation and regeneration processes.

Protoplasts can be isolated from all plant parts *viz.*, leaves, callus, roots, shoot apices, fruits, embryos, microspores, and suspension cultures. But in dicots, leaf mesophyll is the most suited explant for protoplast isolation. In this study, *in vitro* generated leaf explant gave higher protoplast yield and viability compared to callus. Firoozobady and DeBoer (1986) showed that age and growth condition of the donor tissue are very important in regeneration of cell wall and achieving cell division. Also, the most suitable source for protoplast isolation is mesophyll cells of young leaves. Leaves from *in vitro* grown plants were always found to be more promising for protoplast isolation than those of *in vivo* plants (Pavan *et al.*, 2000). Mliki *et al.* (2003) isolated highest number of protoplasts from leaves of 4–5 week old shoots of grapes (*Vitis vinifera*).

During protoplast purification higher protoplast yield (30 x 10^5 protoplasts per g and 10 x 10^5 protoplasts per g) and viability (90.91 per cent and 100 per cent) was obtained with 21.00 per cent sucrose in floatation medium in leaf and callus, respectively. Geetha *et al.* (2000) used 21.00 per cent sucrose for purifying

protoplasts isolated from leaf and calli both in ginger and cardamom. Thomas (2009) purified protoplasts isolated from leaves of *Tylophora indica* using 20.00 per cent sucrose in floatation medium.

Protoplasts are very sensitive to light, therefore they are cultured in dark or diffuse light for the first 4 - 7 days. In this study, microcalli developed, when protoplasts were cultured in dark first and then to diffused light at a plating density of 10×10^5 in treatment PCM_5 (liquid culture medium + 2,4- D 0.50 mg L^{-1} + BAP 0.50 mg L^{-1} + NAA 0.50 mg L^{-1} + mannitol 0.50 M). Visible microcalli colony was observed in about 60 days from callus derived protoplast and 70 days from leaf derived protoplast. Prakash *et al.* (1997) reported development of microcalli in liquid MS medium containing NAA 2.00 mg L^{-1} and BAP 0.50 mg $L^{-1}$by about 45 days and macrocalli in MS gelled medium containing NAA 2.00 mg $L^{-1}$and BAP 0.50 mg L^{-1} by about 65 days in *C. annuum*. Iwamoto and Ezura (2006) reported visible colony formation after one month of culture of cotyledon, hypocotyl and leaf derived protoplasts of *Solanum integrifolium*. Thomas (2009) conveyed that the development of microcalli from mesophyll protoplasts of *Tylophora indica* occurred within 60 days of culture in liquid MS medium with 2,4-D 4 µM, mannitol 0.40 M and sucrose 3.00 per cent.

Future Line of work

- Shoot regeneration medium has to be optimized further to get better regeneration from callus.

- Protocol for macro calli development from protoplast derived micro calli and subsequent plant regeneration has to be standardized.

Summary

6. SUMMARY

The present study entitled "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L. was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-17. The study was carried out in two phases *viz*., establishment of callus culture and organogenesis; and standardization of protocol for protoplast culture.

The objective of the study was to establish callus culture from different explants in *C. frutescens* and to establish protocol for protoplast isolation from callus/leaf mesophyll and to culture protoplast.

Seeds of *C. frutescens* variety Vellayani Samrudhi, obtained from the Department of Olericulture, College of Agriculture, Vellayani were used to raise the *in vitro* seedlings. Leaves and internodes from *in vitro* raised seedlings were used as explants, to study the effect of different auxins (NAA, IAA, IBA, 2,4-D and picloram), cytokinins (BA and Kn) and their combinations in MS medium on callus induction and multiplication.

Among the forty four treatments tried, the best callusing (100 per cent) was obtained in MS media supplemented with picloram 0.50, 1.00, 1.50 and 2.00 mg L^{-1} (C41, C42, C43, and C44), NAA 1.50 mg L^{-1}, NAA 2.00 mg L^{-1} (C3, C4) and BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} (C29). All concentrations of picloram produced soft white profuse calli, NAA produced yellowish green soft friable calli and BA-NAA combination developed hard callus from both the explants.

When leaves were used as explants three levels (1.00, 1.50, 2.00 mg L^{-1} of picloram gave callus multiplication with a growth score of '4' but in 0.50 mg L^{-1} the growth score was '3'. Two levels of NAA (1.5 mg L^{-1} and 2.00 mg L^{-1}) initiated calli

from leaf explants and any of the concentrations of IBA did not induce callusing with the same. But, with internodal explants all levels of picloram produced soft white profuse calli with a growth score of '4'. All the levels of NAA initiated yellowish green soft friable calli with a growth score of '4'. Also, two levels of IBA (1.50 mg L^{-1} and 2.00 mg L-1) initiated soft callus with root initials from internodal explants. MS media with BA-NAA combination initiated similar type of hard callus with growth score '3' from both the explants.

Calli obtained from the best treatments from above experiment were used for organogenesis study. Among the seventy eight hormonal combinations tried for organogenesis, calli from the C29 treatment gave shoot regeneration in R37 (MS + BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1}) and R61 (MS + BA 5.00 mg L^{-1} + IAA 2.00 mg L^{-1}). The treatment R37 initiated shoot on 41st day and attained a shoot length of 5.5 cm with 6 nodes per shoot in 65 days of culture. R61 initiated a shoot in 90 days and recorded a shoot length of 1.5 cm and 2 nodes in 110 days.

The microshoots were transferred to sixteen rooting treatments with varying levels of auxins. The highest rooting per cent (83.33) was obtained in three treatments, Rt7 (MS + IAA 1.50 mg L^{-1}), Rt12 (MS + IBA2.00 mg L^{-1}) and Rt15 (MS + 2,4-D 1.50 mg L^{-1}). The earliest rooting (12.83 days) was observed in the treatment Rt7 with 5.33 roots per shoot and a root length of 4.53 cm.

Leaves excised from *in vitro* raised seedlings and calli produced in MS + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} (C29), were used as explants for protoplast isolation. Leaves and calli were incubated in cell protoplast washing (CPW) solution (pH – 5.8) containing various levels of mannitol, celllase – macerozyme combination for varrying time period in dark with a shaking of 50 rpm at 25 $\pm$ 2 °C to assess the protoplast yield and viability.

Leaf bits incubated in (CPW) solution containing cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.50 M for 6 h (DM_{28}) in dark at 27°C, yielded (124 x 10^5) protoplast per g, with a viability of 95.16 per cent. The callus yielded maximum protoplast (36 x 10^5 protoplasts per g) in an enzyme combination of cellulase 2.00 per cent + macerozyme 0.50 per cent + mannitol 0.60 M after 4 h (DM_{47}) of incubation under same conditions. This is on par with DM_{48} (cellulase 2.00 per cent + macerozyme 0.5 per cent + mannitol 0.60 M; incubation period 6 h) and DM 51 (cellulase 4.00 per cent + macerozyme 0.25 per cent + mannitol 0.60 M; incubation period 2 h).

In protoplast purification, floatation medium containing 3 different levels of sucrose (15, 21, 25 per cent) were tried and was found that floatation medium with 21 per cent sucrose recorded maximum protoplast yield (30 x 10^5 protoplasts per g tissue and 10 x 10^5 protoplasts per g callus) and maximum viability (90.91 per cent and 100 per cent), from leaf derived and callus derived protoplast, respectively.

The purified protoplasts were adjusted to three different levels of protoplast density ($2x10^5$, 5x 10^5,$10x10^5$) and cultured on protoplast culture medium CMI (liquid MS culture media with varying concentrations of mannitol and 2,4- D). The purified protoplasts with 10 $x10^5$ plating density initiated microcalli in liquid MS medium supplemented with mannitol 0.50 M, 2,4- D 0.50 mg L^{-1} and sucrose 30g L^{-1} (PCM5) in 45 days. Further development to visible colony formation of microcalli was obtained on addition of liquid MS medium supplemented with mannitol 0.40 M and sucrose 5g L^{-1}, in 60 days from callus derived protoplast and in 70 days from leaf derived protoplast.

In the study, maximum callusing response was obtained in MS medium with picloram1.50 mg L^{-1}. Organogenesis was obtained from the calli derived in MS medium with BA 3.00 mg $L^{-1}$and NAA 1.00 mg L^{-1}. The shoot initiated from the calli

in MS medium with BA 3.00 mg $L^{-1}$and IBA 1.00 mg L^{-1}. The rooting of microshoots could be obtained in MS medium with IAA 1.50 mg L^{-1}. In protoplast isolation, leaf gave higher protoplast yield and viability in CPW solution with cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.50 M, incubated in dark for 6 h and callus,in CPW solution with cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.60 M, incubated in dark for 4 h. The protoplasts purified in 21 per cent sucrose supplemented floatation medium and adjusted to a plating density of 10 x 10^5, initiated microcalli in liquid MS medium supplemented with mannitol 0.50 M, 2,4- D 0.50 mg L^{-1} and sucrose 30g L^{-1}. The visible colony formation of microcalli was obtained on addition of liquid MS medium supplemented with mannitol 0.40 M and sucrose 5g L^{-1}.

In this study, a callus mediated *in vitro* regeneration system has been established in *C. frutescens*. A protocol has also been developed for protoplast isolation from leaf and calli, and its culture resulting in microcalli formation.

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Appendix

APPENDIX I

Stock solutions of Murashige and Skoog's medium

Constituent	Quantity (mg L^{-1})	Quantity required for preparing the stock (g)	Volume of stock (ml)	Concentration of stock	Volume required for 1L of medium (ml)
Stock A					
NH_4NO_3	1650	16.5	250 ml	40 x	25 ml
KNO_3	1900	19.0			
$MgSo_4.7\ H_2O$	370	3.7			
KH_2PO_4	170	1.7			
Stock B					
$CaCl_2.2H_2O$	440	8.8	100 ml	200 x	5 ml
Stock C					
H_3BO_3	6.2	0.62	100 ml	1000 x	1 ml
$MnSo_4.4\ H_2O$	22.3	1.69			
$Na_2MoO_4.2\ H_2O$	0.25	0.25			
KI	0.83	0.083			
$ZnSo_4.7\ H_2O$	8.6	0.860			
Stock D					
$Na_2EDTA2HO$	37.3	0.745	100 ml	200 x	5 ml
$FeSo_4.7H_2O$	27.8	0.556			
Stock E					
$CuSo_4.5H_2O$	0.025	0.125	250 ml	2000 x	0.5 ml
$COCl_2.6H_2O$	0.025	0.125			
Stock F					
Glycine	2	0.2			
Nicotinic acid	0.5	0.05	100 ml	1000 x	1 ml
Pryridoxine-HCl	0.5	0.05			
Thiamine-HCl	1	0.01			

Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L.

by

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ABSTRACT

The present study entitled "Establishment of *in vitro* regeneration systems from callus and protoplast in *Capsicum frutescens* L. was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The objective of the study was to establish callus culture from different explants in *C. frutescens*, to establish protocol for protoplast isolation from callus/leaf mesophyll and to culture protoplast. The study was carried out in two phases *viz.*, establishment of callus culture and organogenesis; and standardization of protocol for protoplast culture.

Callus was induced from leaves and internodal segments from *in vitro* raised seedlings. Among the 44 treatments in MS medium with different combinations of auxins (NAA, IAA, IBA, 2,4-D and picloram) and cytokinins (BA and Kn), 100 per cent callus induction was obtained in MS media supplemented with picloram 0.50, 1.00, 1.50 and 2.00 mg L^{-1} (C41, C42, C43, and C44), NAA 1.50 mg L^{-1}, NAA 2.00 mg L^{-1}(C3, C4) and BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} (C29). Among the 78 treatments tried for organogenesis, calli obtained from C29 treatment showed organogenesis in MS + BA 3.00 mg L^{-1} + IBA 1.00 mg L^{-1} (R37) and (MS + BA 5.00 mg L^{-1} + IAA 2.00 mg L^{-1} (R61) in 41 and 90 days, respectively. The microshoots obtained recorded 83.33 per cent rooting in MS medium supplemented with IAA 1.50 mg L^{-1}(Rt7) in 12.83 days.

Leaves excised from *in vitro* seedlings, and calli produced in MS + BA 3.00 mg L^{-1} + NAA 1.00 mg L^{-1} (C29), were used as explants for protoplast isolation. Leaf bits incubated in cell protoplast washing (CPW) solution containing cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.50 M (maintained at pH 5.8) for 6 h (DM_{28}) in dark at 27°C, yielded (124 x 10^5) protoplast per g, with a viability of 95.16 per cent. The callus yielded maximum protoplast (36 x 10^5 protoplasts per g) in an enzyme combination of cellulase 2.00 per cent + macerozyme 0.50 per cent + mannitol 0.60 M (maintained at pH 5.8) after 4 h

(DM_{47}) of incubation under same conditions. In protoplast purification, floatation medium with 21 per cent sucrose recorded maximum protoplast yield (30 x 10^5 protoplasts per g tissue and 10 x 10^5 protoplasts per g callus) and maximum viability (90.91 per cent and 100 per cent), from leaf derived and callus derived protoplast, respectively.

The purified protoplasts with 10 x10^5 plating density initiated microcalli in liquid MS medium supplemented with mannitol 0.50 M, 2,4- D 0.50 mg L^{-1} and sucrose 30g L^{-1} (PCM_5) in 45 days. Further development to visual colony formation of microcalli was obtained on addition of liquid MS medium supplemented with mannitol 0.40 M and sucrose 5g L^{-1}, in 60 days from callus derived protoplast and in 70 days from leaf derived protoplast.

In the study, maximum callusing response was obtained in MS medium with picloram 1.50 mg L^{-1}. Organogenesis was obtained from the calli derived in MS medium with BA 3.00 mg L^{-1} and NAA 1.00 mg L^{-1}. The shoot initiated from the calli in MS medium with BA 3.00 mg L^{-1} and IBA 1.00 mg L^{-1}. The rooting of microshoots could be obtained in MS medium with IAA 1.50 mg L^{-1}. In protoplast isolation, leaf gave higher protoplast yield and viability in CPW solution with cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.50 M, incubated in dark for 6 h and callus, in CPW solution with cellulase 2.00 per cent, macerozyme 0.50 per cent and mannitol 0.60 M, incubated in dark for 4 h. The protoplasts purified in 21 per cent sucrose supplemented floatation medium and adjusted to a plating density of 10 x 10^5, initiated microcalli in liquid MS medium supplemented with mannitol 0.50 M, 2,4- D 0.50 mg L^{-1} and sucrose 30g L^{-1}. The visual colony formation of microcalli was obtained on addition of liquid MS medium supplemented with mannitol 0.40 M and sucrose 5g L^{-1}.

In this study, a callus mediated *in vitro* regeneration system has been established in *C. frutescens*. A protocol has also been developed for protoplast isolation from leaf and calli, and its culture resulting in microcalli formation.