Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto

Ambreena Din
(2012-A-918-M)

Division of Floriculture, Medicinal & Aromatic Plants

Faculty of Horticulture

Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

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Ambreena Din
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O our lord! Do not make us responsible if we forget or make mistake

O our lord! Do not lay on us burden such as you laid on those before us

O our lord! Do not impose upon us that which we have not the strength

Pardon us

Forgive us

And have mercy upon us

You are our lord – master

So help us against unbelieving people

(Al –Baqarah: 286)

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Floriculture, Medicinal & Aromatic Plants,
Shalimar Campus Srinagar – 190 025

Certificate – I

This is to certify that the thesis entitled, **"Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Horticulture (Floriculture & Landscape Architecture)**, to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Ambreena Din (Regd. No. 2012-A-918-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

(Dr. Imtiyaz T. Nazki)
Chairman
Advisory Committee

Endorsed

Head,
Division of Floriculture, Medicinal & Aromatic Plants

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Floriculture, Medicinal & Aromatic Plants,
Shalimar Campus Srinagar – 190 025

Certificate – II

We, the members of the Advisory Committee of **Ms. Ambreena Din (Regd. No. 2012-A-918-M),** a candidate for the degree of **Master of Science in Horticulture (Floriculture & Landscape Architecture)**, have gone through the manuscript of the thesis entitled, **"Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

Advisory committee

Chairman
Dr. Imtiyaz T. Nazki
Associate Professor, Division of FMAP,
SKUAST-Kashmir, Shalimar

Members

Dr. Neelofar
Associate Professor, Division of FMAP,
SKUAST-Kashmir, Shalimar

Dr. M.A. Mir
Assistant Professor, Division of Fruit Science,
SKUAST-Kashmir, Shalimar

Dr. S.A. Mir
Head, Division of Agri-Statistics,
SKUAST-Kashmir, Shalimar

Dean PG Nominee
Dr. Imtiyaz Murtaza
Associate Professor, Division of PHT,
SKUAST-Kashmir, Shalimar

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Floriculture, Medicinal & Aromatic Plants,
Shalimar Campus Srinagar – 190 025

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This is to certify that the thesis entitled, **"Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto"** submitted by **Ms. Ambreena Din (Regd. No. 2012-A-918-M)** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Horticulture (Floriculture & Landscape Architecture)**, was examined and approved by the Advisory Committee and External Examiner on …….

Chairman **External Examiner**
Advisory Committee

Head,
Division of Floriculture, Medicinal & Aromatic Plants

Dean,
Faculty of Horticulture, SKUAST-Kashmir

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Floriculture, Medicinal & Aromatic Plants, Shalimar
190 025

Name of the student : **Ambreena Din**

Registration No. : 2012-A-918-M

Major subject : Floriculture and Landscape Architecture

Minor subjects : Plant Physiology/Fruit Science

Major advisor : **Dr. Imtiyaz T. Nazki**
Associate Professor,
Division of Floriculture, Medicinal &
Aromatic Plants, SKUAST-K, Shalimar

Title of the Thesis : **"Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto"**

ABSTRACT

The experiment entitled Studies on *in vitro* Propagation in *Alstroemeria* cv. Pluto was conducted to standardize protocol for aseptic establishment, callus induction, proliferation, and rooting from rhizome tips, rhizome sections, shoot tips, shoot nodal segments and inflorescence buds. Highest culture asepsis of 79.20 per cent at 2 weeks of culture and 68.08 per cent at 4 weeks of culture was recorded in rhizome tips following sterilization treatment with Carbendazim 200 ppm for 30 minutes + $HgCl_2$ (0.1 %) dip for 10 minutes and final treatment with ethyl alcohol (70 %) for 1 minute. Rhizome tips and rhizome section explants survived sterilant treatment better than other explants. MS-liquid medium supplemented with BAP + IBA: 1.5 + 0.2 mg l^{-1} proved best for culture establishment (89.42 %) in case of rhizome tips and (56.13 %) in case of rhizome sections. MS-solid medium with plant growth regulator combinations BAP + IBA: 1.0 + 0.2 mg l^{-1} fortified with activated charcoal resulted in an establishment of (78.25 %) in rhizome tips and (40.24 %) in case of rhizome sections. Callus induction was highest in MS-solid medium fortified with BAP + NAA: 0.5 + 4.0 mg l^{-1}. Rhizome tips cultured on MS-medium BAP + IBA + GA_3 + Activated

charcoal: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1} gave highest proliferation (88.85 %) along with highest number of erect shoots (5.75) , number of new rhizome buds (3.75), rhizome fresh weight/shoot complex (6.05), and multiplication index (2.76). Highest Rooting (54.81 %) along with lowest days to appearance of root (10.87), highest number of roots (3.12) and highest root length (16.42 mm) was recorded in MS-liquid medium fortified with NAA 1.5 mg l^{-1}.

Keywords : Tissue culture, *in vitro* propagation, Alstroemeria, rhizome tips

Signature of Student

Dated:_____

Signature of Major Advisor

Dated:_____

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Place: Shalimar, Srinagar
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List of Abbreviations

Abbreviation	Full form
AC	Activated charcoal
BAP	6-Benzyl amino purine
BA	6-Benzyladenine
2, 4-D	2, 4-dichlorophenoxyacetic acid
GA_3	Gibberelic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog's (1962) medium
NAA	Naphthalene acetic acid
PGRs	Plant growth regulators
Psi	Pounds per square inch
TDZ	Thidiazuron
µm	Micro molar (concentration)

Chapter – 1

INTRODUCTION

Alstroemeria also known as Inca lily, lily of Incas or Peruvian lily, is a rhizomatous monocot belonging to the family *Alstroemeriaceae*. *Alstroemeria* hybrids are very popular cut flowers occupying position within top ten cut flowers of the world due to diversity of colours, low energy requirement and longer vase life. Alstroemeria are also grown as bedding or potted plants (Bridgen, 1997) and has a premium potential as a cut flower crop in temperate and sub temperate areas of the country especially of Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh, Uttaranchal, West Bengal and Nillgiri hills of Tamil Nadu. The increase in production area and the introduction of new hybrids by breeding have necessitated development of efficient methods for cloning, since modern Alstroemeria hybrids are often sterile (triploids) and can only be propagated vegetatively. Alstroemeria are conventionally propagated vegetatively by rhizome division but it is inefficient, time consuming, requires large area for maintaining stock plants and contributes to the spread of viral diseases. Globally, the demand for clean healthy plant materials for agriculture, horticulture, forestry and ornamental industries is in excess of 16 trillion units per year, which equals US$ 4 trillion. For ornamentals, it is estimated that the global sale of cut flowers and pot plants is US$ 90 and 60 billion, respectively. The production of ornamentals by commercial micropropagation was in 1986, 130 million plants globally. Over one billion ornamentals are produced yearly through micropropagation (Prakash, 2009).

Alstroemeria plants consist of a sympodial fleshy, multi-stemmed rhizome from which shoots and fibrous roots arise. Some of the fibrous roots latter become thickened storage roots as the plants develop. The storage roots are called *'Radices Medullosae'*. The shoots can be either reproductive or vegetative depending upon the environmental conditions. *Alstroemeria* shoots produce a whorled cymose inflorescence. Each cyme is sympodially branched with up to

five florets that open one after another. The sexual parts of the flowers are dichogamous. The stamens open first and shed pollen before the stigma becomes receptive. The flowers come in a variety of colours. Post-harvest life is terminated by petal drop or yellowing of the leaves or both.

There are several commercial cultivars of *Alstroemeria* available. Van Scheepen (1991) gave descriptions of species and the common ones include *A. aurea*, *A. brasiliensi*, *A. caryophyllaea*, *A. chiliensis*, *A. ligtu*, *A. pelegrina*, *A. psittacina* etc. Among the cultivated *Alstroemeria*, *Ligtu* hybrids (LH) have originated from a natural crossing between *A. ligtu* and *A. ligtu spp. Simsii* (Robinson, 1963). They are widely used for cut flowers in Japan. On the other hand *A. pelegrina* var. *rosea* (PR) is dwarf and has large flowers. The interspecific hybrids between LH and PR have also been produced (Ishikawa *et al.,* 1997).

Alstroemeria is a relatively recent introduction into the world's floriculture scene and has become a major cut flower. It is also used as a potted flowering plant for the home decoration and as an herbaceous landscape plant in the mild climates. Plants have routinely survived in the Netherlands and in Maryland (Zone-6) in the United States. Cultivars are patented and growers must sign agreements. Division is not legal unless authorized.

In India *Alstroemeria* is a recent introduction. The crop was introduced in 2001 by Ministry of Agriculture, GOI, under Food and Agriculture Organization programme at three Model Floriculture Centers in India-Ooty (Tamil Nadu), Chial (Himachal Pradesh) and Srinagar (Jammu & Kashmir). The crop was introduced in SKUAST-Kashmir, Shalimar in 2005-06 under ICAR sponsored Horticulture Mini Mission-I. Initial results in the poly house and open conditions were promising.

Attempts have been made by few workers to multiply Alstroemeria *in vitro* through rhizome tips (Hussey *et al*., 1979; Gabryszewska and Hempel, 1985; Lin and Monette, 1987; Chiari and Bridgen, 2000) but contamination was the

major bottleneck to start the cultures. Alstroemeria can be started with apical shoot tips but there is also a strong dominance effect suppressing the growth of axillary buds (Bond and Alderson, 1993b).

Several components and combinations of compounds interfere with the mechanism of apical dominance, thereby releasing buds from their inhibited state like Tri-iodobenzoic acid, an anti-auxin (Niedergang and Skoog, 1956; Rubery, 1987) and Thidiazuron (TDZ), having a cytokinin like mode of action (Mok *et al*., 1982).

In vitro multiplication of elite plant genotype is the widely used commercial application of plant biotechnology and offers immense opportunities to multiply more number of disease free planting material in a shortest possible time (Lin *et al*., 2000).

Alstroemeria is generally propagated by rhizome division of the three year old plants. However, multiplication rate is low. Propagation through seed is not commonly practiced due to variability in hybrids and long and difficult germination. Legal planting material imported from foreign breeder companies is still very costly ranging from Rs. 500-600/plant. Cultivars introduced in late nineties in India are currently out of the patent regime. However, planting material is not easily available due to constraints imposed on multiplication rate under conventional propagation methods of rhizome division. This has been the single major stumbling block that has prevented *Alstroemeria* from becoming widely adopted by cut flower growers in Kashmir and Himachal Pradesh. There is a need to establish *in vitro* propagation protocols for the cultivars already available with research institutes and Government departments. This shall not only ensure availability of quality planting material for growers but also open up avenues for cultivar improvement through *in vitro* mutagenesis. Keeping in view the importance of *Alstroemeria* as a lower energy requirement cut flower crop for

growers in Kashmir the current study was conducted with the following objectives:

i) Standardization of disinfection protocol for aseptic establishment.

ii) Identification of suitable explant for establishing *in vitro* cultures in *Alstroemeria*

iii) Standardization of media and growth regulator regimes for callus induction, callus proliferation and organogenesis if any.

iv) Development of protocol for multiplication and rooting of *Alstroemeria in vitro*.

Chapter – 2

REVIEW OF LITERATURE

Results obtained by different workers on *in vitro* propagation of *Alstroemeria* as well as other related crops with similar problems have been reviewed under the following sub-heads.

2.1 Explant

2.2 Surface sterilization of explant

2.3 Establishment and proliferation of cultures

2.4 Callus induction and regeneration

2.5 Rooting

2.1 Explant

2.1.1 Inflorescence stem

Different types of explants have been employed to establish cultures. Ziv *et al.* (1973) used young actively growing tissue explants from *Alstroemeria* inflorescence stem taken at a distance of 1-2 mm below the apex that proved capable of regenerating buds and roots from which small plantlets could be established.

2.1.2 Rhizome

Lin and Monette (1987) regenerated plantlets from rhizome tips cultured on solid and liquid media based on Murashige and Skoog salt formulation. The quality of the cultures was superior when intact rather than longitudinally sliced rhizome tips were used as explants, and when a temperature of 8° rather than 22°C was used at the initiation stage.

Hakkaart *et al.* (1988) used a technique for elimination of *Alstroemeria* mosaic virus from infected *Alstroemeria* cultivars in which meristems were excised from rhizome tips and placed on a medium containing indole-3-butyric

acid at a cultivar dependent concentration. Pierik *et al.* (1988), used terminal and lateral tips from fleshy rhizomes that were isolated *in vitro* and induced to form new rhizomes.

Elliott *et al.* (1993) established *Alstroemeria cv*. Parigro Pink rhizome tip explants. Bond and Alderson (1993), Gabryszewska (1995) successfully cultured rhizome apical and axillary tips cultured on Murashige and Skoog medium with BA at 2 mg l^{-1} and NAA at 0.5 mg l^{-1}.

Gabryszewska and Hempel (1985), Han *et al*. (1994), Chiari and Bridgen (1996, 2000) cultured rhizome pieces of Alstroemeria for *in vitro* plantlet regeneration.

Yousef *et al*. (2007) compared the regeneration ability of plantlets using *in vitro* and *in vivo* grown rhizome buds as explants that were cultured on MS basal medium with 3 different compositions of growth regulators (1, 0.2 mg l^{-1} NAA with 1 mg l^{-1} BA and 0.2 mg l^{-1} IAA with 1 mg l^{-1} BA) and the cultures were incubated in 18 + or -1 degrees C at 16 h photoperiod.

Pumisutapon *et al.* (2009) prepared four types of explants - an intact rhizome with two intact shoots (+R+2S), an intact rhizome with two decapitated shoots (+R-2S), a decapitated rhizome with two intact shoots (-R+2S), and a decapitated rhizome with two decapitated shoots (-R-2S), *in vitro* to study the apical dominance in *Alstroemeria*. Rhizomes of pale orange-coloured *Alstroemeria cv*. 'Caralis' were used as the source of explants by Amir *et al*. (2012).

2.1.3 Leaf

Lin *et al.* (1997), Jang *et al*. (1999), Lin *et al*. (2000) reported that leaves of Alstroemeria can be used as an explant to enhance the multiplication efficiency. Lin *et al*. (1998) induced direct shoot regeneration from leaf explants of *Alstroemeria* clone VV2406, a selection from a tetraploid breeding line. Explants contained a leaf blade and a small portion of stem node, which were cut from

erect shoots of *in vitro*-multiplicated plantlets. Shoot regeneration capacity of the excised leaf explants was significantly related to the position of the explant on the stem. The youngest explant which was located closest to the shoot apex gave the highest response. Khaleghi and Azadi (2011) selected vegetative explants (node, internode, and leaf) and employed embryogenic calluses for gene transformation in *Alstroemeria* and studied the embryogenic callus induction in *Alstroemeria* cv. Fuego.

2.1.4 Somatic Embryos

Gonzalez and Alderson (1990) obtained a callus from mature embryos of cv. Butterfly on MS medium supplemented with either 2 or 4 mg picloram l^{-1} or 4 mg 2, 4-D l^{-1} combined with benzyladenine (BA) or kinetin (0-4 mg l^{-1}). Shoots regenerated and torpedo-like structures (somatic embryos) formed when callus was transferred to regeneration media containing BA plus picloram or 2, 4-D. Gonzalez and Alderson (1992) cultured excised somatic *Alstroemeria cv.* Butterfly embryos on solid MS medium alone, or on MS medium supplemented with 0.1 mg BA l^{-1} + 10% (v/v) coconut water or 0.1 mg GA_3 l^{-1}. Cultures were incubated at 25 or 15°C and the number of embryoids which developed into single shoot plantlets was generally higher at 15 than 25°C. After 4 weeks of culture, the greatest percentage of cultures with single shoot plantlets (25%) was obtained from the medium supplemented with GA_3 and cultured at 15°C, but the greatest percentage of cultures with callus (75%) was obtained from the same medium at 25°C. Hutchinson *et al*. (1994) obtained callus when mature zygotic embryos were cultured on MS medium supplemented with 20 µM kinetin and 10 or 20 µM NAA. Callus that was transferred to MS medium supplemented with 20 µM kinetin and 20 µM NAA for long term culture, maintained a regeneration capacity of 40 per cent over an 8 month period. Schaik *et al*. (1996) studied the plant regeneration ability of callus obtained from zygotic embryos of diploid *Alstroemeria* inodora and a tetraploid cultivar. The best explants for somatic

embryogenesis were immature zygotic embryos in half-ovules when the endosperm was soft and white.

2.1.5 Root, stem segments, shoot tips, rhizome buds

Gonzalez and Alderson (1995) attempted Callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and GA_3. The best results were obtained with mature embryos and after 18 days in culture, callus was produced on 40, 34 and 32 per cent of embryos cultured on MS medium supplemented with 4 mg 2, 4-D l^{-1}, 2 mg picloram l^{-1} and 4 mg picloram l^{-1}, respectively.

2.1.6 Leaf, stem, rhizome, inflorescence apices

Pedraza-Santos *et al*. (2006) developed a protocol for the *in vitro* regeneration of *Alstroemeria cv.* 'Yellow King', using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices) and various temperature and light/dark regimes, hormone and salt concentrations and several hormone concentrations for shoot multiplication and rooting and found that only the young floral apices produced adventitious shoots by direct organogenesis. The highest shoot induction rate (10.4 shoots per explant) was obtained by incubation in the dark for 15 days at 8 ^{o}C followed by 15 days at 25 ^{o}C and a 16-h/8-h light/dark regime, on a Murashige and Skoog (1962) liquid medium at 50 per cent of the salt concentration, supplemented with 2.5 mg l^{-1} Kinetin, 1.5 mg l^{-1} BA and 1.0 mg l^{-1} NAA, using a piece of filter paper to support the explant. The highest shoot multiplication rate (9 shoots per explant) was obtained on a liquid MS medium at full strength supplemented only with BA at 1.0 mg l^{-1}.

2.1.7 Shoots

Fujita *et al*. (2010) observed high rhizome-formation ability but frequent contamination with soil microorganisms *in vitro* when apical meristem explant

taken from underground shoots of 3-4 cm length that had sprouted from the rhizome. Apical meristem cut from vegetative shoots of 50-100 cm length had hardly any rhizome-formation ability, and these were not suitable as explants for micro propagation.

2.1.8 Leaf, Node and Internode

Kim *et al*. (2006) obtained high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) in *Alstroemeria* from nodes with axil tissue cultured first on a Murashige and Skoog (MS) medium supplemented with 10 µM thidiazuron and 0.5 µM indole-3-butyric acid and after that on a Schenk and Hildebrandt (SH) medium supplemented with 9.1 µM 2, 4-dichlorophenoxy acetic acid and 2.2 µM benzylaminopurine (BA). Both types of callus were maintained on modified MS medium supplemented with 20.8 µM picloram. Regenerated plants were established in the greenhouse and flowered normally. Khaleghi and Azadi (2008) studied the plant regeneration ability of callus in the ornamental monocot *Alstroemeria cv*. Fuego. High frequency (23%) of compact callus induction was obtained on a Schenk and Hildebrandt (SH) medium supplemented with 2 mg l^{-1} picloram from nodal segments excised from plants grown in the greenhouse and also employed embryogenic calluses for gene transformation in *Alstroemeria* and studied the embryogenic callus induction in *Alstroemeria* cv. Fuego. The vegetative explants (node, internode, leaf) along with various concentrations of auxins (picloram, NAA, IAA, 2, 4-D) either with or without BAP were taken into experimentation. The nodal explants provided the highest embryogenic calluses. The internodal explants resulted in a lower embryogenic callus production than the nodal ones. The leaf explants did not prove suitable for callus induction. The highest induction rate of embryogenic calluses was obtained for ½ MS medium supplemented with 2 mg l^{-1} of NAA. As for regeneration, callus was transferred to regeneration medium supplemented with 2 mg l^{-1} of BAP (Khaleghi and Azadi, 2011).

Seyyedyousefi *et al.* (2013) used segments of nodes and internodes of *Alstroemeria cv*. Fuego as explant that were cultured in MS basal medium with different concentrations of BAP (0.0 and 0.5 mg l^{-1}) and NAA (0.0, 1.0 and 2.0 mg l^{-1}) to produce callus.

2.1.9 Other Related Crops

Shetty *et al.* (1982) cultured single sprouting buds of Turmeric on modified MS medium containing sucrose (40 g l^{-1}) and kinetin (0.2-0.5 mgl^{-1}). Nathan *et al*. (1993) used axillary and terminal buds of Heliconia Psittacorum as a source of explant. Tissue blocks of approximately 1 cm size containing either the apical bud or axillary buds were excised from shoots and after surface sterilization further trimmed to approximately 3 mm size before culturing. In ginger (*Zingiber officinale* Rosa.) the newly emerging buds are the favourite explant for initiating the cultures. Hosoki and Sagawa (1997) took the rhizome buds of ginger and cultured on modified MS medium. Nel (1985) established cultures with shoot-tips of ginger. Meristem of size 0.1-0.5 mm were excised from pale yellow sprouted buds of ginger for establishment of cultures (Bhagyalakshmi and Singh, 1988).

Malamug *et al.* (1991) while working with *ginger cv*. Kintoki used 1-2 mm shoot tip explants. Devi and Nayar (1993) used shoot tips as explants excised from one and three months old suckers. Sharma and Singh (1995) advocated that shoot tips of ginger can be regenerated into plantlets. Similarly, De Him and De Paez (1998) used shoot tips of ginger for *in vitro* multiplication. Arimura *et al.* (2000) took basal portion of ginger as an explant and cultured on MS medium containing different concentration of NAA.

2.2 Surface sterilization of explant

Hakkart and Versluijs (1985) initially rinsed the rhizomes of *Alstroemeria cv's* 'Rosario', 'Toledo' and 'Jubilee' with tap water and later dipped for few seconds in 70 per cent alcohol. Final sterilization was done in a laminar flow

cabinet with 5 per cent Ca-hypochlorite followed by rinsing 3 times with sterile water.

Pierik (1987) identified four source of infection during *in vitro* propagation of *Alstroemeria* viz. the plant (internal as well as external), the nutrient medium (insufficiently sterilized), the air and the aseptic work. The rhizome tip explant regenerated profusely but contamination was found to be a major bottleneck. Since rhizome tips grow below the surface of soil and it was quite difficult to disinfect and also reported that prior to sterilization. *Alstroemeria* rhizomes are to be washed under tap water to remove soil etc. Later rhizome segments are to be surface sterilized initially in 70 per cent ethanol for 2-3 seconds followed by 20min dip in 1.5 NaOCl (with few drops of Tween-20) and rinsed thrice in sterile tap water (Pierik *et al*., 1988).

Lin and Monette (1987) obtained best results with the treatment of rhizome tips of *Alstroemeria cv.* 'Alsaan' with 0.6 per cent sodium hypochlorite plus 0.1 per cent Tween-20 for 20 minutes with continuous stirring followed by rinsing 3 times in sterile distilled water.

Dekker's *et al*. (1991) used 1 per cent potassium hypochlorite for 20 minutes for the disinfection of axillary buds obtained from rhizomes (*Zingiber officinale, Curcuma amada, C. domestica*), aerial stem nodes (*Costus* spp.) and bulbils (*Alpinia parpurata*) arising from inflorescence.

Pedersen and Brandt (1992) developed a procedure for the disinfection of *Alstroemeria* rhizome tips based on scale leaves immersed for 1-10 min in 3 per cent Korsolin or 1 per cent NaOCl.

Jyothi *et al*. (2008) reported use of 1.0 per cent mercuric chloride for 8 minutes in ginger a plant with a underground rhizome similar in architecture as *Alstroemeria.*

Sathyagowri and Seran (2011) also reported reasonable level of culture asepsis and survival with Carbendazim (0.3%) + Doxycycline (0.2%) for 10

minutes followed by 70% ethanol for 1 minute in ginger rhizome expants with buds.

Seyyedyousefi *et al*. (2013) also used fragments of stem containing node and internode and washed thoroughly under running tap water for 20 minutes and disinfected with 1.5 per cent NaOCl aqueous solution for 15 minutes.

2.3 Establishment and proliferation of cultures

Ziv *et al*. (1973) established cultures of Alstroemeria from inflorescence segments equally on White's medium and Murashige and Skoog's medium. A higher ratio of auxin to cytokinin resulted in root regeneration, while the reverse ratio promoted bud differentiation. Plantlets were obtained from bud subculture on a low sucrose medium supplemented with IAA but without kinetin and also used young actively growing tissue explants from *Alstroemeria* inflorescence stem taken at a distance of 1-2 mm below the apex that proved capable of regenerating buds and roots from which small plantlets could be established.

Gabryszewska and Hempel (1985) recommended the use of BA for tissue multiplication and used MS medium containing NaFeEDTA 40.3 mg l^{-1}, mesomositol 100 mg l^{-1}, thiamine 4 mg l^{-1} and different concentrations of plant growth regulators for initial establishment of cultures of Alstroemeria. Maximum shoot multiplication was obtained on MS medium supplemented with BA (8 mg l^{-1}).

Hakkart and Versluijs (1985) established cultures from meristem tips in *Alstroemeria cv.* 'Rosario' on MS medium containing 2-ip [isopentenyladenine] 0.01 mg l^{-1} and NAA 1 mg l^{-1} in addition to thiamine 0.4 mg l^{-1}, sucrose 3 % and Difeo Bacto agar 0.6 per cent.

Pierik *et al*. (1988) used terminal and lateral tips from fleshy rhizomes that were isolated *in vitro* and induced to form new rhizomes and studied the influence of temperature, light, rhizome portion, plant growth regulators, length of multiplication cycle, media and saccharose concentration on mean number and

length of upright growing shoots as well as on rhizome multiplication rate (RMR). A temperature of 15 ^{o}C was ideal for increasing the number of elongated growing shoots whereas, 21 ^{o}C was optimal for increasing the length of shoots as well as significantly increased rhizome multiplication rate. Saccharose 3-4 per cent, BA @ 3-4 mg l^{-1}, and a multiplication cycle of 3 weeks given 5 times, was most optimal for rhizome multiplication.

Bond and Alderson (1993a) assessed the effects of mechanical and chemical methods of removing or reducing apical dominance on the multiplication of *Alstroemeria* cultivars Valiant, Parade and Eleanor grown *in vitro* and observed significantly enhanced rhizome multiplication on sub culturing rhizome explants without aerial shoots and rhizome apices, and rhizome explants divided into single internodes with or without aerial shoots, but sub culturing rhizome explants with only the aerial shoot or rhizome apices removed had no significant effect.

Bond and Alderson (1993b) observed that for good multiplication the requirements for the culture environment were a temperature of 15°C, an irradiance of 5 W m^{-2} with a day length of 8 hours.

Elliott *et al.* (1993) established *Alstroemeria cv*. Parigro Pink rhizome tip explants on modified MS medium with P added as KH_2PO_4 at 0, 0.01, 0.05, 0.25, 1.25 or 2.5 mM and cultures were transferred to fresh media every 4 weeks. Explants supplied with 1.25 or 2.5 mM P produced significantly more shoots and growing points, and greater FW of rhizomes and shoots, than those provided with lower P concentrations. Shoot tissue P concentrations > 7 mmol kg^{-1} FW were required for maximum *in vitro* growth of *Alstroemeria cv.* Parigro Pink.

Han *et al.* (1994) cultured rhizome tips of *Alstroemeria hybrid cultivars* 'Othello', 'Lilac Glory', 'Cyprus' and Yellow prince' on MS medium supplemented with various growth regulators and obtained best results for rhizome multiplication on MS medium containing 1.0-2.0 mg BA + 0.3 mg l^{-1} IAA or 0.2 mg NAA l^{-1}. Apical rhizomes produced more branched

rhizomes/explant than did lateral rhizomes. Further continuous lighting at 3000 lux resulted in greater shoots and rhizomes formation than did 16 hours lighting or culture in darkness.

Gabryszewska (1995, 1996) successfully cultured rhizome apical and axillary tips cultured on Murashige and Skoog medium with BA at 2 mg l^{-1} and NAA at 0.5 mg l^{-1} and observed the presence of BA in the medium markedly increased the number of upright growing shoots and more shoots at 25°C than at 17°C. The highest number of lateral rhizomes was observed on a medium containing 60 or 80 g sucrose l^{-1} and BA. Presence of BA in the medium markedly influenced the formation of upright growing shoots; the tallest shoots were found in cultures on media containing 20 or 30 g sucrose l^{-1}. Low and high concentrations of sucrose inhibited the formation and elongation of upright growing shoots.

Lin *et al*. (1997) advocated a two-step protocol for the induction of shoots from leaf explants of *Alstroemeria*. Leaf explants with stem node tissue induced best results when cultured initially for 10 days on MS medium containing 10 µM Thidiazuron and 0.5 µM indole butyric acid followed by several subculturing on regeneration medium containing 2.2 µM BAP.

Podwyszynska *et al.* (1997) developed an effective micro propagation method for new Polish cultivars of *Alstroemeria cv*. Juanita, and *in vitro* experiments were conducted to improve the efficiency of multiplication and rooting stages. Rhizomes were cultured on media containing 1.5, 3 or 6 mg BAP [benzyladenine] l^{-1}. The greatest number of aerial shoots and shortest roots, but the poorest rhizome rooting ability, was observed at 6 mg BAP l^{-1}.

Lin *et al*. (1998) induced direct shoot regeneration from leaf explants of *Alstroemeria* clone VV2406, a selection from a tetraploid breeding line. Explants contained a leaf blade and a small portion of stem node, which were cut from erect shoots of *in vitro* multiplicated plantlets. The youngest explant which was

located closest to the shoot apex gave the highest response. A gradient response toward the shoot apex was observed in percentage of shoot regeneration and in the number of shoots/regenerating explant.

Jang *et al.* (1999) investigated the efficiency of shoot induction from leaf explants, and the subsequent development of shoots into complete plants with rhizomes and developed a good regeneration system applicable for micro propagation in *Alstroemeria* culture conditions. The youngest ex-plant located close to the shoot apex gave rise to the highest regeneration rate.

Lin *et al.* (2000) micro propagated six tetraploid *Alstroemeria* clones by rhizome multiplication, and within a 3-week subculture interval, the average rhizome multiplication rate for all genotypes was 2.3.

Chiari and Bridgen (2000) investigated the growth of *in vitro Alstroemeria* hybrids through morphological studies and produced rhizome halves that regenerates by cutting the rhizome with a horizontal or vertical longitudinal cut.

Chiari and Bridgen (2002) excised stem apical meristems, rhizome apical meristems and rhizome axillary meristems from *Alstroemeria* plants and were grown *in vitro* on modified Murashige and Skoog (MS) media containing different concentrations of Gibberelic acid and 6-benzylaminopurine [benzyladenine] (BA). Plantlets developed from stem apical meristems never regenerated a rhizome and eventually died but the highest regeneration rate (74.1%) of plantlets with a rhizome was observed when rhizome axillary meristems were grown on modified MS medium containing 8.9 µM of BA.

Pedraza-Santos *et al.* (2006) developed a protocol for the *in vitro* regeneration of *Alstroemeria* cv. 'Yellow King', by testing for shoot induction, using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices), temperature and light/dark regimes, hormone and salt concentrations and tested several hormone concentrations for shoot multiplication and rooting and found that only the young floral apices produced adventitious

shoots by direct organogenesis. The highest shoot induction rate (10.4 shoots per explant) was obtained by incubation in the dark for 15 days at 8 ^{o}C followed by 15 days at 25 ^{o}C and a 16-h/8-h light/dark regime, on a Murashige and Skoog (1962) liquid medium at 50 per cent of the salt concentration, supplemented with 2.5 mg l^{-1} Kinetin, 1.5 mg l^{-1} BA and 1.0 mg l^{-1} NAA, using a piece of filter paper to support the explant. The highest shoot multiplication rate (9 shoots per explant) was obtained on a liquid MS medium at full strength supplemented only with BA at 1.0 mg l^{-1}.

Yousef *et al*. (2007) compared the regeneration ability of plantlets using *in vitro* and *in vivo* grown rhizome buds as explants. *In vitro* and *in vivo* grown rhizome buds were cultured on MS basal medium with 3 different compositions of growth regulators (1, 0.2 mg l^{-1} NAA with 1 mg l^{-1} BA and 0.2 mg l^{-1} IAA with 1 mg l^{-1} BA) and the cultures were incubated in 18 + or -1 ^{0}C at 16 h photoperiod. Four subcultures of explants were done on the same fresh media with 3 weeks intervals.

Khaleghi *et al.* (2008) in his experiment, used lateral and terminal buds of rhizomes (4-6 mm) that were cultured on solidified MS medium containing 30 g l^{-1} agar supplemented with different concentrations of BAP and NAA after surface disinfection and sub cultured every three weeks. The greatest number of shoots was obtained from the medium supplemented with 1.5 mg BAP l^{-1} and 0.2 mg NAA l^{-1}.

Pumisutapon *et al.* (2009) prepared four types of explants-an intact rhizome with two intact shoots (+R+2S), an intact rhizome with two decapitated shoots (+R-2S), a decapitated rhizome with two intact shoots (-R+2S), and a decapitated rhizome with two decapitated shoots (-R-2S), *in vitro* to study the apical dominance in *Alstroemeria*.

Hutchinson *et al.* (2010) observed the effect of Thidiazuron, NAA, and BAP on *in vitro* propagation of *Alstroemeria aurantiaca cv.* Rustica from shoot tip explants.

Successful micro-propagation of *Alstroemeria* in liquid medium using slow release of medium components was done by Klerk and Brugge (2010) in which *Alstroemeria* rhizomes were micro-propagated on semi-solid medium and in liquid medium. In liquid medium, growth was much enhanced (ca. 70%).

Fujita *et al*. (2010) observed high rhizome-formation ability but frequent contamination with soil microorganisms *in vitro* when apical meristems explant taken from underground shoots of 3-4 cm length that had sprouted from the rhizome and also the apical meristem cut from vegetative shoots of 50-100 cm length had hardly any rhizome-formation ability, and these were not suitable as explants for micropropagation. Apical meristems of floral shoots that sprouted in the field were cultured on MS medium containing 0.01 mM BAP, 0.001 mM NAA, and 3% sucrose and a large number of rhizome buds were propagated by subculturing on a medium containing 6% sucrose, 0.01 mM BAP, and 0.001 mM NAA. Rhizome buds were subcultured in a culture vessel with ½ N-MS medium containing 9% sucrose, 0.01 mM BAP, and 0.001 mM NAA.

Pumisutapon *et al*. (2011) studied apical dominance in *Alstroemeria* and used rhizome as the standard explant with a tip and two vertically growing shoots from which the larger part had been excised leaving ca. 1 cm stem. The axillary buds that resumed growth were located at this 1-cm stem just above the rhizome. They were released by removal of the rhizome tip and the shoot tips. Replacement of excised tips by lanolin with indole-3-butyric acid (IBA) restored apical dominance. The auxin transport inhibitors 2, 3, 5-triiodobenzoic acid (TIBA) and N-1-napthylphthalamic acid (NPA) reduced apical dominance. 6-Benzylaminopurine (BAP) enhanced axillary bud outgrowth but the highest concentrations (>9 µM) caused fasciation.

2.3.1 Other related crops

Hosoki and sagawa (1977) successfully cultured buds of ginger on MS medium consisting MS major elements. Pillai and Kumar (1982) established and multiplied ginger cultures on Schenk and Hildebrandt medium and obtained 15 daughter shoots per original shoot in 3 months. In case of turmeric, Shetty *et al.* (1982) cultured buds on MS medium containing sucrose (40 g l^{-1}) and Kinetin (2.5 mg l^{-1}). After 1-2 subculturing callus produced numerous buds which later on developed into plantlets.

Illahi and Jabeen (1987) induced callus in ginger on ½ strength MS media containing different combination of growth regulators and obtained regeneration on MS medium containing 2,4-D. Sato *et al*. (1987) obtained satisfactory *in vitro* shoot formation in ginger on Gamborge B5 obtained on MS medium.

Bhagyalakshmi and Singh (1988) induced shoots from meristems in ginger when cultured on MS medium containing sucrose 6%, coconut milk 20%, ascorbic acid 100ppm, glutamine 400 ppm, activated charcoal 250 ppm, BA 0.5 ppm, IBA 0.4 ppm and agar 0.8%. Meristems derived shoots exhibited consistent multiplication on this medium. Further, liquid media was less effective than solid medium for micro-propagation.

Inden *et al*. (1988) cultured shoot tips of ginger on MS basal medium supplemented with 5 mg l^{-1} IBA, 0.5 mg l^{-1} NAA and obtained 4 shoots of 20 to 30 mm size from an explant within 6 weeks.

Devi and Nayer (1993) obtained successful establishment and multiplication of shoot tips of banana on MS medium consisting inositol (5.5 µM), thiamine HCl (2.97 µM), BA (22 µM), sucrose (12 µM) and coconut water (15 %).

Sharma and Singh (1997) developed disease free clones (7.7 shoots/bud) of *Zingiber officinale* by cutting active buds on MS medium supplemented with Kinetin 2 mg l^{-1} and sucrose 20 g l^{-1}.

Arimura *et al*. (2000) obtained adventitious shoots in ginger on MS medium containing Kinetin 25 µM. Roots were induced on growth regulator free MS medium. Kim *et al*. (2000) induced callus in ginger on N6 medium supplemented with 2 mg l^{-1} NAA and further obtained plantlets regeneration from callus on MS medium supplemented with BA 1.2 mg l^{-1}

Archana *et al*. (2013) developed improved micro propagation protocol for *Zingiber moran* and *Z. zerumbet*, two wild species of the genus *Zingiber* and tested the effects of growth regulators, sugar concentrations, and nutrients on the rate of shoot initiation and multiplication and observed an increase in proliferation and multiplication that occurred in modified Murashige and Skoog (MS) medium supplemented with benzyladenine and kinetin and also observed that about 2 % sucrose and 0.7 % agar to be the optimum for shoot multiplication and regeneration.

2.4 Callus induction and regeneration

Gonzalez and Alderson (1990) obtained callus from mature embryos of *cv*. Butterfly on MS medium supplemented with either 2 or 4 mg picloram l^{-1} or 4 mg 2,4-D l^{-1} combined with benzyladenine (BA) or kinetin (0-4 mg l^{-1}). Shoots regenerated and torpedo-like structures (somatic embryos) formed when callus was transferred to regeneration media containing BA plus picloram or 2, 4-D. Shoots were also produced by somatic embryos upon transfer to other media.

Gonzalez and Alderson (1992) cultured excised somatic *Alstroemeria cv.* Butterfly embryos on solid MS medium alone, or on MS medium supplemented with 0.1 mg BA l^{-1} + 10% (v/v) coconut water or 0.1 mg GA_3 l^{-1}. Cultures were incubated at 25 or 15°C and the number of embryoids which developed into single shoot plantlets was generally higher at 15 than 25° C. After 4 weeks of culture, the greatest percentage of cultures with single shoot plantlets (25%) was obtained from the medium supplemented with GA_3 and cultured at 15°, but the greatest

percentage of cultures with callus (75%) was obtained from the same medium at 25°C.

Hutchinson *et al*. (1994) obtained callus when mature zygotic embryos were cultured on MS medium supplemented with 20 µM kinetin and 10 or 20 µM NAA. Callus that was transferred to MS medium supplemented with 20 µM kinetin and 20 µM NAA for long term culture, maintained a regeneration capacity of 40% over an 8 month period.

Gonzalez and Alderson (1995) attempted callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and GA_3. The best results were obtained with mature embryos and after 18 days in culture, callus was produced on 40, 34 and 32% of embryos cultured on MS medium supplemented with 4 mg 2, 4-D l^{-1}, 2 mg picloram l^{-1} and 4 mg picloram l^{-1}, respectively. Callus fresh weight increased at a higher rate over the next 45 days in the medium supplemented with 2 mg picloram/litre than in the other 2 media.

Schaik *et al*. (1996) studied the plant regeneration ability of callus obtained from zygotic embryos of diploid *Alstroemeria* inodora and a tetraploid cultivar. The best explants for somatic embryogenesis were immature zygotic embryos in half-ovules when the endosperm was soft and white. Nodular embryogenic callus was induced on callus induction medium with a success rate of 54%. The best callus induction period was 10 weeks. Somatic embryos were formed after transfer of the callus to regeneration medium. These somatic embryos had the typical features of zygotic *Alstroemeria* embryos.

Hutchinson *et al*. (1997) cultured Embryogenic callus induced from mature zygotic embryos on MS medium supplemented with 40 µM NAA and 20 µM kinetin, and used as inoculum for liquid cultures. When transferred to a semi-

solid, half-strength MS medium supplemented with casein hydrolysate, cell aggregates successfully differentiated into plantlets which later grew to maturity under greenhouse conditions.

Lin *et al*. (1997) developed a 2-step protocol for the induction of shoots from *Alstroemeria* (genotype VV024) leaf explants with stem node tissue attached were incubated on shoot induction medium for 10 days, and then transferred to regeneration medium. Shoots from the area adjacent to the region between the leaf base and node tissue regenerated within 3 weeks after transfer, without a callus phase. The best induction was obtained with Murashige and Skoog medium containing thidiazuron (10 µM) and IBA (0.5 µM). The regeneration medium contained 6-benzylaminopurine [benzyladenine] (2.2 µM) and after several subcultures of leaf explants with induced shoots, normal plantlets with rhizomes were formed.

Akutsu and Sato (2002) developed an efficient procedure for plant regeneration from calluses of *Alstroemeria* by somatic embryogenesis. Suspension cells were maintained in Murashige and Skoog's (MS) medium supplemented with 1 mg l^{-1} picloram and then used for either solid or liquid culture. Friable embryogenic calluses formed in liquid half-strength MS medium supplemented with 0.5 mg l^{-1} naphthalene acetic acid (NAA) and 0.5 mg l^{-1} benzyl adenine (BA). The friable calluses developed pro-embryos after transfer to solidified half-strength MS medium without growth regulators and described an efficient procedure for transformation of calli of the monocotyledonous plant *Alstroemeria* by Agrobacterium rhizogenes. Inoculated calli were plated on medium that contained cefotaxime to eliminate bacteria. Four weeks later, transformed cells were selected on medium that contained 20 mg l^{-1} hygromycin. Plants derived from transformed calli were produced on half-strength MS medium supplemented with 0.1 mg l^{-1} GA_3 after about 5 months of culture (Akutsu and Sato, 2004).

Kim *et al.* (2001) concluded that the nodal explants can be successfully used as a source for transformation in combination with the MS medium containing 1 mg l^{-1} 2, 4-D, 0.25 mg l^{-1} BAP, 3% sucrose (w/v) and 0.75% (w/v) micro agar for the production of a high level of compact callus and somatic embryos and in (2005) developed an efficient system for the regeneration of plants from protoplasts in *Alstroemeria.* Friable embryogenic callus (FEC) proved to be the best source for protoplast isolation and culture when compared with leaf tissue and compact embryogenic callus. Micro-calluses were formed after 4 week of culture. Ninety per cent of the micro-calluses developed into FEC after 12 week of culture on proliferation medium. FEC cultures produced somatic embryos on a regeneration medium and half of these somatic embryos developed shoots(Kim *et al.,* 2005) and also obtained high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) in *Alstroemeria* from nodes with axil tissue cultured first on a Murashige and Skoog (MS) medium supplemented with 10 µM thidiazuron and 0.5 µM indole-3-butyric acid and after that on a Schenk and Hildebrandt (SH) medium supplemented with 9.1 µM 2, 4-dichlorophenoxy acetic acid and 2.2 µM benzylaminopurine (BA). Both types of callus were maintained on modified MS medium supplemented with 20.8 µM picloram (Kim *et al.*, 2006).

Khaleghi and Azadi (2008) obtained a high frequency (23%) of compact callus induction in *Alstroemeria cv*. Fuego on a Schenk and Hildebrandt (SH) medium supplemented with 2 mg l^{-1} picloram from nodal segments excised from plants grown in the greenhouse. After three months of culture, compact embryogenic calluses (CECs) were transferred to the modified Murashige and Skoog (MS) medium supplemented with 5 mg l^{-1} picloram for further proliferation of CECs.

In an experiment on vegetative explants (nodes, internodes and leaves) on *Alstroemeria cv.* 'Feugo', Khaleghi and Azadi (2011) used various concentrations

of auxins (Picloram, NAA, IAA, 2, 4-D) with or without BAP and reported that nodal explants provided highest embryogenic calluses.

Amir *et al.* (2012) evaluated the number of growth regulators as well as supplements to the MS-basal medium on the regeneration of *Alstroemeria* rhizome explants.

Seyyedyousefi *et al.* (2013) evaluated the effect of explant type and plant growth regulators (NAA and BAP) on callus formation of *Alstroemeria cv*. Fuego and showed that the explants source and different concentrations of growth regulators influenced callus production. Segments of nodes and internodes were cultured in MS basal medium with different concentrations of BAP (0.0 and 0.5 mg l^{-1}) and NAA (0.0, 1.0 and 2.0 mg l^{-1}) to produce callus and observed that node was better explant than internode to produce callus best at 0.5 mg l^{-1} of BAP and 2.0 mg l^{-1} of NAA.

Violeta *et al.* (2013) reported, callus induction with high auxin:cytokinin ratio from floral explants in Iris on MS medium + 2.0 mg l^{-1} TDZ and 3.0 mg l^{-1} NAA.

2.5 Rooting

Ziv *et al*. (1973) advocated a higher ratio of auxin to cytokinin for *in vitro* root regeneration in *Alstroemeria.*

Gabryszewska and Hempel (1985) recommended the use of BA for tissue multiplication and NAA (1.0-16.0 mg l^{-1}) for rooting.

Lin and Monette (1987) regenerated plantlets from rhizome tips cultured on solid and liquid media based on Murashige and Skoog salt formulation. The quality of the cultures was superior when intact rather than longitudinally sliced rhizome tips were used as explants, and when a temperature of 8° rather than 22°C was used at the initiation stage. More roots were produced on rhizome tips containing a rhizome apical meristem than on rhizome sections lacking such a

meristem and 90 per cent of the rooted plantlets were successfully acclimatized and developed into true-to-type flowering plants.

Hakkart and Versluijs (1988) reported that *Alstroemeria cv.* 'Rosario' rooted when *in vitro* regenerated shoots were cultured on MS medium containing IAA (1 mg l^{-1} or NAA 2 mg l^{-1}). Out of 32 cultures, 12 rooted cultures were obtained and observed that root formation was better on filter paper bridges in a liquid medium than on a solid medium. Subsequent transfer into soil was more successful with the plantlets rooted in liquid medium than that with those rooted on solid medium.

Pierik *et al*. (1988) conducted *in vitro* rooting experiments in *Alstroemeria cv.* Toledo and reported that MS medium containing NAA 0.5 ppm in addition to saccharose 5 per cent was most ideal. Further rooting was promoted by a day length of 18h in comparison to 8h and an irradiance of 7 W/m^2. Initial dark treatments had a negative effect and optimal rooting occurred at 21 ^{0}C in comparison to 25^o and 27 ^{o}C.

Han *et al.* (1994) successfully cultured rhizome tips of the hybrid cultivars 'Othello', 'Lilac Glory', 'Cyprus' and 'Yellow Prince' on MS medium supplemented with various growth regulators. The most effective (for percentage rooting, number of roots and branched rhizomes/explant and root length) was IBA at 3.0 mg l^{-1}.

Gonzalez and Alderson (1995) observed no root formation when experiments were attempted on callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and/or GA_3.

Pedersen *et al*. (1996) investigated flower induction by testing 9 genotypes at four temperature regimes (5, 10, 15 or 20°C for 6 weeks) and three methods of propagation (by seeds, rhizome division or micro propagation) and for micro

propagated plants, temperature treatments were carried out during root formation *in vitro*.

Podwyszynska *et al.* (1997) observed that the greatest number of aerial shoots and shortest roots, but the poorest rhizome rooting ability, at 6 mg BAP l^{-1} and used rhizome cultures of Polish *Alstroemeria X hybrida cv*. Juanita to enhance the effectiveness of a micro propagation method for new cultivars and selections. The effects of cytokinins (benzyl adenine, kinetin and 2iP), auxins (IAA, IBA and NAA) and growth retardants (paclobutrazole and flurprimidol), alone or in combination, were studied in relation to rhizome branching, aerial shoot production and rhizome rooting. Application of BA at low concentration with paclobutrazole (0.1-0.5 mg l^{-1}) or flurprimidol (0.01-0.1 mg l^{-1}) in the presence of 1 mg NAA/litre resulted in a high number of aerial shoots (5-6, but these were shorter) and higher rooting ability of the rhizomes. Growth retardants applied with NAA strongly stimulated root formation but suppressed their elongation (Podwyszynska *et al*., 1998).

Kristiansen *et al*. (1999) observed a synergistic promoting effect of PPFD (Photosynthetic photon flux density) and sucrose on root formation. Root formation after transfer to rooting medium was affected by sucrose and PPFD during the multiplication phase. PPFD did not influence root formation after propagation on 7% sucrose, whereas on 3 or 5% sucrose root formation was gradually inhibited when PPFD was decreased below 17 µmol m^{-2} s^{-1}. The formation of thick roots was promoted by propagation in light but not influenced by sucrose concentration. Root formation on rooting medium was reduced by BA and promoted both by NAA and high levels of sucrose. Thick roots were only produced in the presence of NAA and not affected by sucrose treatment.

Chiari and Bridgen (2000) produced plants with the split technique that rooted and flowered regularly, and were true-to-type.

Pedraza-Santos *et al*. (2006) developed a protocol for the *in vitro* rooting of shoots on a liquid MS medium, either with or without plant hormones using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices), temperature and light/dark regimes, hormone and salt concentrations and several hormone concentrations for rooting.

2.5.1 Other related crops

Successful rooting of *in vitro* regenerated shoots of ginger was obtained within 2 months when the shoots were transferred to basal MS medium supplemented with BA 1 ppm (Hosoki and Sagawa, 1977). Pillai and kumar (1982) could induce root hair formation in ginger shoots by prolonged culturing on establishment/multiplication medium. However, to induce more root hair development, daughter plantlets were subcultured on blotting paper bridges in liquid medium.

Bhagyalakshmi and Singh (1988) found that prolonged culturing on multiplication medium resulted into root hair formation in case of ginger. However, unrooted shoots can be induced to root by sub culturing in the quarter strength MS medium supplemented with sucrose (3 5), ascorbic acid (100 mg l^{-1}), activated charcoal (100 mg l^{-1}) and agar 8 per cent. This medium did not require any growth regulator for root induction.

Malamug *et al*. (1991) regenerated the shoots from callus in *ginger cv.* 'Kintoki' and concluded that the multiplication medium containing NAA (1 mg l^{-1}) and BA (5 mg l^{-1}) was optimal to produce satisfactory root system.

Nathan *et al*. (1993) obtained *in vitro* rooting in *Heliconia psittacorum* on hormone free medium. A high (92 %) rooting was observed when MS medium was supplemented with thiamine HCl (0.5 ppm), myoinositol (100 ppm), sodium hydrogen phosphate (170 ppm), adenine sulphate (80 ppm), gelrite (2 g l^{-1}) and sucrose (3 %).

Chang and Criley (1993) could induce roots in *in vitro* regenerated shoots of ornamentals pink ginger (*Alpinia purpurata*) within 4 weeks on agar solidified on half strength medium with 2 per cent sucrose.

Dogra *et al.* (1994) rooted excised rhizome buds of elite lines of ginger on MS medium supplemented with BA 2.5 mg l^{-1} and NAA 0.5 mg l^{-1}. The maximum number of roots were formed on MS medium supplemented with NAA (1 ppm).

Huang (1995) regenerated *in vitro* plantlets with complete root system directly from shoot tips (20.2-0.9 mm in length) of ginger on MS medium containing BA 2.0 mg l^{-1} and NAA 0.6 mg l^{-1}. Babu *et al.*(1996) developed profuse callus in ginger on MS medium supplemented with 2,4-D 1mg l^{-1}alone or 2,4-D (0.5 mg l^{-1}) + BA (1 mg l^{-1}) and found that individual embryoids developed into plantlets with better rooting when NAA (1 mg l^{-1}) was added to culture medium.

Rout and Das (1997) obtained rooting in *in vitro* regenerated shoots on half strength MS supplemented with IBA or IAA and 2 per cent sucrose.

Archana *et al*. (2013) developed improved micro propagation protocol for *Zingiber moran* and *Z. zerumbet*, two wild species of the genus *Zingiber* and observed that Naphthalene acetic acid at 0.5 mg l^{-1} produced the best rooting response for both the species. Regenerated plantlets were acclimatized successfully and cytogenetic stability was confirmed by RAPD profiling and ploidy checks.

Chapter – 3

MATERIALS AND METHODS

The present investigation on studies on *in vitro* propagation in *Alstroemeria cv.* "Pluto" was carried out in the Biotechnology Laboratory of the Division of Pomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar during 2012-2014.

The outline of the methods and technical programme of different experiments has been described under the following heads:

3.1 Materials (stock plants, chemicals, activated charcoal, glassware, and Culture media)

3.2 Methods

- 3.2.1 Preparation of stock solutions
- 3.2.2 Preparation of stock solution of growth regulators
- 3.2.3 Preparation of culture media (solid and liquid)
- 3.2.4 Cleaning of glassware
- 3.2.5 Sterilization of equipment, and culture media
- 3.2.6 Culture environment, culture incubation
- 3.2.7 Collection of explant

3.3 Experimental details

- 3.3.1 Experiment-1: Standardisation of disinfection protocol for various explants in *Alstroemeria*
- 3.3.2 Experiment-2: Standardization of media and growth regulator combination for culture establishment in *Alstroemeria*
- 3.3.3 Experiment-3: Standardisation of callus induction and organogenesis in *Alstroemeria*
- 3.3.4 Experiment-4: Standardisation of shoot proliferation in *Alstroemeria*
- 3.3.5 Experiment-5: Standardisation of rooting in *Alstroemeria*

3.4 Statistical analysis

3.1 Materials (stock plants, chemicals, activated charcoal, glassware and Culture media)

3.1.1 Stock Plants

An Orange coloured cultivar of *Alstroemeria* hybrid grown in the green houses of floriculture field, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar was selected for conducting the present investigation.

3.1.2 Chemicals

Compounds containing major and minor elements, sucrose, agar, and organic compounds like amino acids, vitamins and plant growth substances, activated charcoal required for preparation of media during the present investigation were procured from Hi-media, India.

3.1.3 Glassware

The borosilicate glassware used in the study was procured from Borosil. Glassware was washed with detergent followed by thorough washing with tap water. The glassware was then dried in hot air oven at 100 ºC for 24 hours before use.

3.1.4 Culture media

Murashige and Skoog (1962) medium was employed during the course of present investigation (Table 3.1). In addition to above, some modifications in Murashige and Skoog (1962) medium were also employed details of which have been provided in the appropriate sections. Both solid and liquid media was prepared during the present investigation.

3.1.5 Liquid Media

Different materials like wattman filter paper, cotton, sterilized gauze were procured for the preparation of the liquid media during the period of investigation.

Table 3.1 : Chemical composition of Murashige and Skoog (1962) medium

Components	Chemical formula	Quantity(mg l^{-1})
Macro-nutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride, dihydrate	$CaCl_2.2H_2O$	330
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	370
Potassium phosphate, monobasic	KH_2PO_4	170
Micro-nutrients		
Potassium iodide	KI	0.83
Boric Acid	H_3BO_3	6.2
Manganese sulphate, tetra hydrate	$MnSO_4.4H_2O$	22.3
Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6
Sodium molybdate, dihydrate	$Na_2MoO_4.2H_2O$	0.25
Cupric Sulphate, pentahydrate	$CuSO_4.5H_2O$	0.025
Cobaltous chloride, hexahydrate	$CoCl_2.6H_2O$	0.025
Ethylenediamine-tetra-acetic acid disodium salt	$Na_2EDTA.2H_2O$	37.3
Ferrous sulphate, heptahydrate	FeSO4.$7H_2O$	27.8
Organic supplements		
Myo inositol	$C_6H_{12}O_6$	100.00
Glycine	$C_2H_5NO_2$	2.00
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine hydrochloride	$C_8H_{11}NO_3.HCl$	0.5
Thiamine hydrochloride	$C_{12}H_{17}CIN_4SO.HCl$	0.1

3.2 Methods

3.2.1 Preparation of stock solutions

Different types of stock solutions of macro elements, micro elements and organics were prepared for each medium and stored in reagent bottles for use. Usually four types of stock solutions (A-D) are made for Murashige and Skoog basal medium (1962). However, making of seven types of stock solutions (A, B, C, D, E, F, and G) of Murashige and Skoog (1962) is prevalent in the Biotechnology Lab of Division of Pomology and the same was followed in the current investigation (Table 3.2). During the entire investigation period, 200 ml of each stock solution was prepared each time which was sufficient for making 20 litres of medium. For making one litre of medium, 10 ml of each stock solution was added to 700 ml of double distilled water in a beaker and final volume made to one litre by adding double distilled water.

Seven stock solutions were used in the preparation of MS medium. Stock solution (A/B/D) containing only one chemical was prepared by dissolving required quantity of each chemical (quantity of chemical required for making 20 litres of medium) in 150 ml of double distilled water and final volume made to 200 ml with double distilled water. Thus stock solution with a strength of 100 X was obtained. Stock solution containing more than one chemical (C/E/F/G) was prepared by weighing the required quantity of each chemical (quantity of chemical required for making 20 litres of medium) separately and dissolved to the last particle in double distilled water followed by mixing them together slowly with continuous stirring. Final volume of 200 ml of the mixture was made by addition of double distilled water. Stock solutions were stored in corning reagent bottles at 4^{o}C.

Table 3.2: Composition and preparation of stock solutions for Murashige and Skoog (1962) medium

Stock	Components	Chemical formula	Qty. mg l^{-1}	Qty. for 20 litres	Qty. of stock solution	Conc. of stock solution
A	Ammonium nitrate	NH_4NO_3	1650	33.00 g	200 ml	100 X
B	Potassium nitrate	KNO_3	1900	38.00 g	200 ml	100 X
C	Potassium iodide	KI	0.83	16.6 mg	200 ml	100 X
	Boric acid	H_3BO_3	6.2	124 mg		
	Potassium di-hydrogen ortho-phosphate	KH_2PO_4	170	3.4 g		
	Sodium molybdate	$Na_2MoO_4.2\ H_2O$	0.25	5 mg		
	Cobaltous chloride	$CoCl_2.6H_2O$	0.025	0.5 mg		
D	Calcium chloride, dihydrate	$CaCl_2.2H_2O$	330	6.6 g	200 ml	100 X
E	Magnesium sulphate, heptahydrate	$MgSO_4.7H_2O$	370	7.4 g	200 ml	100 X
	Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6	172 mg		
	Manganese Sulphate, tetra hydrate	$MnSO_4.4H_2O$	22.3	446 mg		
	Cupric Sulphate pentahydrate	$CuSO_4.5H_2O$	0.025	0.5 mg		
F	Ethylene diaminetetra acetic acid disodium salt	$Na_2EDTA•2H_2O$	37.3	746 mg	200 ml	100 X
	Ferrous Sulphate heptahydrate	FeSO4.$7H_2O$	27.8	556 mg		
G	Glycine	-	2.00	40 mg	200 ml	100 X
	Nicotinic acid	-	0.5	10 mg		
	Pyridoxine HCl	-	0.5	10 mg		
	Thiamine HCl	-	0.1	2 mg		

The required quantity of cobalt chloride was too small (0.5 mg) to be weighed properly on the available digital balance. This quantity was multiplied by 100 and the quantity obtained (50 mg) was dissolved in 100 millilitres of water. Then one millilitre of this solution was added to stock solution C and final volume of 200 ml was made by adding double distilled water**.** Similar method was used for adding required quantity of cupric sulphate and thiamine to their respective stock solutions.

For the preparation of the iron stock solution (stock solution F), required quantities of $FeSO_4.7H_2O$ and Na_2.EDTA were dissolved separately in 75 ml double distilled water. Na_2.EDTA solution was heated over hot plate and stirred to dissolve it completely. It was then added to $FeSO_4.7H_2O$ solution gently and stirred well to get a homogeneous light yellow solution. Final volume (200 ml) of the mixture was made by adding double distilled water and stored in an amber-coloured reagent bottle.

3.2.2 Preparation of stock solutions of growth regulators

Basal medium was supplemented with different types of growth regulators. Small quantity of Stock solution (50 ml) of each growth regulator was prepared on weekly/monthly basis and stored in refrigerator at 4^oC. Stock solutions of benzyl-amino purine (BAP), kinetin, naphthalene-acetic-acid (NAA), Gibberelic acid (GA) were prepared by dissolving 50 mg of each growth regulator in 25 ml of 0.50 N NaOH and then volume was made to 50 ml with double distilled water. However, 2, 4-D, indole-3-aceticacid (IAA) and indole-3-butyric-acid (IBA) were prepared by dissolving 50 mg of each growth regulator well with some alcohol drops and then volume was made to 50 ml with double distilled water.

3.2.3 Preparation of solid culture media

The required quantity of sucrose (usually 30 g l^{-1}) and myoinositol (0.1 g l^{-1}) was dissolved in double distilled water in a beaker. Required quantity of stock

solutions containing macro elements, microelements, vitamins, growth regulators and activated charcoal were added as per the treatment requirement. Final volume of the medium was made with the addition of double distilled water. The pH of medium solution was adjusted at 5.7 by adding 1N NaOH or 1 N HCl drop wise with a micropipette. Care was taken that electrode does not come in direct contact with the walls of beaker and bulb of electrode remains completely immersed in the solution. The medium was slightly heated in microwave oven and then required quantity of agar was added, stirred well with scalpel or stirrer and again heated in microwave oven to boil so as to dissolve the agar completely. The medium was allowed to cool for few minutes and subsequently dispensed in culture vessels. Non absorbent cotton plugs covered with news paper were used to plug the culture vessels.

3.2.4 Preparation of liquid culture media

The required quantity of sucrose (usually 30 g l^{-1}) and myo-inositol (0.1 g l^{-1}) was dissolved in double distilled water in a beaker. Required quantity of stock solutions containing macro elements, microelements, vitamins, growth regulators, activated charcoal were added as per the treatment requirement. Final volume of the medium was made with the addition of double distilled water. The pH of medium solution was adjusted at 5.7 by adding 1N NaOH or 1 N HCl drop wise with a micropipette. The medium was dispensed in culture vessels containing Filter Paper Bridge over non-absorbent cotton or sterilized gauze for the support of explant in the culture vessels. Non-absorbent cotton plugs covered with newspaper were used to plug the culture vessels.

3.2.5 Sterilization of culture media

Culture media in test tubes/flasks was sterilized by autoclaving in a horizontal autoclave at 121°C and 1.05 kg cm^{-2} (15 psi) for 20 minutes. This high temperature not only kills bacteria and fungi, but also their heat-resistant spores. Flasks and test tubes were placed in autoclave when it had developed the required

pressure. Lid of the autoclave was closed and vacuum valve opened to remove air from the autoclave chamber. Vacuum valve was closed and steam inlet valve opened. Pressure indicator lamp (green lamp) switches off when required pressure (15 psi) is developed inside the autoclave chamber. Time for autoclaving was counted from the switching off of the pressure indicator lamp of the autoclave. After autoclaving the test tubes and flasks for the required time, electric supply of the autoclave was switched off and exhaust valve opened to release the pressure slowly. Autoclaved media was allowed to cool overnight at room temperature and used when required but within 10 days.

3.2.6 Culture Environment

All the aseptic manipulations like surface sterilization, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laboratory using laminar air flow cabinet where pure air is forced out continuously so that no contaminant is allowed to enter the cabinet during working time. Pure air is generated by forcing the air to pass through a series of fine filters which remove dust and other potential contaminants. The working table of laminar air flow chamber was first surface sterilized with absolute alcohol and then by ultraviolet light for 30 minutes. The forceps, scalpel and Petri-dishes were first steam sterilized in an autoclave at 121^{o}C for 20 minutes. These instruments were further sterilized at the start of inoculation by pouring some alcohol over them in the laminar hood followed by flaming. During inoculation forceps and blade were flame sterilized several times. Hands were cleaned and wiped with Hi-Care disinfectant gel before working.

3.2.7 Culture incubation

The cultures were generally incubated at 24±1^{o}C in an air conditioned culture room with a 16/8 hour light/dark regime and a light intensity of 3500 lux.

3.2.8 Collection of explants

Different types of explants that were tried during the course of present investigation were collected from plants maintained at green houses in Floriculture fields, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar. The details regarding collection of different explants are as follows.

3.2.9 Rhizome tips and rhizome sections

Healthy plants of the *Alstroemeria cv.* "Pluto" were dug out from the field with the help of spade and disease free rhizome tips and rhizome portions were placed in clean flasks containing distilled water and brought to the laboratory for further processing.

3.2.10 Leaves, nodal segments and inflorescence buds, shoot tips

Disease free leaves, nodal segments, inflorescence buds, shoot tips were separated from the mother plant with the help of a cutter. The leaves were cut into smaller pieces and placed in clean flasks containing distilled water and brought to the laboratory for further processing.

3.3 Experimental parameters

A variety of experiments were conducted during the present investigation aimed at the development of a protocol for *in vitro* propagation of *Alstroemeria cv.* "Pluto". Details regarding the methodologies adopted are given in the sections that follow.

3.3.1 Experiment-1: Standardisation of disinfection protocol for various explants in *Alstroemeria*

Explant surface sterilization is the first vital step for the initiation of cultures *in vitro.* Final steps in surface sterilization were performed under the aseptic conditions of laminar flow hood. Knives and forceps were flame sterilized before use. While making incisions, knives and forceps were flame sterilized as and when thought necessary to avoid spread of contamination between different

parts of the explant. Details of surface sterilization procedures for different explants are given in the following sections.

3.3.1.1 Rhizome tips and rhizome sections

Explants on arrival in the laboratory were washed with running tap water to remove any adhering dust and debris and cut into manageable size pieces. Afterwards the explants were put in a Tween-20 surfactant solution (few drops) along with required quantity of fungicides and given a vigorous shake for 30 minutes to ensure thorough cleaning. There after the surfactant along with the fungicides was washed off under running tap water followed by a final washing with single distilled water. The specimens were latter brought to laminar flow hood for treatment with mercuric chloride 0.1%, ethyl alcohol 70% and aseptic inoculation. The details of composition of different sterilant formulations and duration of application are given in Table 3.3.

3.3.1.2 Shoot tips and nodal segments

Shoot tips and nodal segments were procured from greenhouse grown plants. These are separated with a sharp scalpel. Explants on arrival in the laboratory were washed with running tap water and thereafter given a vigorous shake for 15 minutes in Tween- 20 surfactant solution fortified with appropriate fungicide and antibiotic concentration. The surfactant and fungicide/antibiotic solution was latter removed by placing the explant under running tap water and a final 2-3 rinses with single distilled water. The details regarding the composition of various sterilant formulations and duration of application are given in the Table 3.3.1.

Table 3.3 : Composition of sterilant formulations and duration of application for surface sterilization of rhizome tips and rhizome portions in *Alstroemeria cv*. Pluto

Details of the Treatment	
S_1	Mercuric chloride dip for 5 minutes
S_2	Mercuric chloride dip for 10 minutes
S_3	Ethyl alcohol dip (70%) for 30 seconds
S_4	Ethyl alcohol dip (70%) for 1 minute
S_5	Bavistin 200 ppm for 30 minutes followed by S_1 and S_3
S_6	Bavistin 200 ppm for 30 minutes followed by S_2 and S_4
S_7	Bavistin 400 ppm for 30 minutes followed by S_1 and S_3
S_8	Bavistin 400 ppm for 30 minutes followed by S_2 and S_4

Table 3.3.1 : Composition of sterilant formulations and duration of application for surface sterilization of nodal segments, leaves, inflorescence buds, shoot tips in *Alstroemeria cv*. Pluto

S_1	Mercuric chloride 0.1% for 3 minutes
S_2	Mercuric chloride 0.1% for 5 minutes
S_3	Bavistin 200 ppm for 15 minutes + Mercuric chloride 0.1% for 3 minutes
S_4	Bavistin 200 ppm for 15 minutes + Mercuric chloride 0.1% for 5 minutes
S_5	Bavistin 400 ppm for 15 minutes + Mercuric chloride 0.1% for 3 minutes
S_6	Bavistin 400 ppm for 15 minutes + Mercuric chloride 0.1% for 5 minutes

3.3.1.3 Observations

Data on following parameters were recorded during the course of investigation.

1. Per cent asepsis after 2 weeks and 4 weeks of culture
2. Per cent survival after 4 weeks of culture

Number of cultures per treatment : 12

Number of replications : 04

Experimental design : Completely Randomized Design

3.3.2 Experiment II: Standardization of media and growth regulator combinations for culture establishment in *Alstroemeria*

Explant surface sterilized with optimal concentration of sterilant were established on MS medium (solid, solid with activated charcoal and liquid) containing different concentrations of plant growth regulators. 250 ml flasks were used for conducting this experiment. The details of experiment are given in the Table 3.3.2.

3.3.2.1 Observations

1) Per cent established cultures:

Recorded as number of cultures showing elongation of shoots or sprouting of buds from rhizome portion**.**

2) Days to rhizome sprouting

Recorded as days to start of shoot elongation and rhizome initiation.

3) Number of sprouted buds

Number of cultures per treatment : 12

Number of replications : 04

Experimental design : Completely Randomized Design

3.3.3 Experiment-III: Standardisation of shoot proliferation in *Alstroemeria*

After the establishment phase, rhizome tips with at least one axillary bud were taken and placed on basal MS media (solid, solid with activated charcoal) consisting different combinations of Plant growth regulators (Table 3.3.3).

Table 3.3.2: Plant growth regulator combinations tried for culture establishment in *Alstroemeria cv*. Pluto

S. No.	MS + PGR's (mg l^{-1})				
	BAP	**IBA**	**NAA**	**Kinetin**	**Activated charcoal**
1.	0.2	0.02	-	-	-
2.	0.4	0.02	-	-	-
3.	0.6	0.02	-	-	-
4.	0.8	0.02	-	-	-
5.	1.0	0.02	-	-	-
6.	1.0	0.2	-	-	-
7.	1.5	0.2	-	-	-
8.	1.5	0.4	-	-	-
9.	1.5	0.6	-	-	-
10.	2.0	0.2	-	-	-
11.	2.0	0.4	-	-	-
12.	2.0	0.6	-	-	-
13.	2.5	0.2	-	-	-
14.	2.5	0.4	-	-	-
15.	2.5	0.6	-	-	-
16.	2.5	0.5	-	-	-
17.	3.0	0.5	-	-	-
18.	0.5	-	-	-	-
19.	1.0	-	-	-	-
20.	1.5	-	-	-	-
21.	-	0.2	-	0.5	-
22.	-	0.2	-	1.0	-
23.	-	-	0.2	0.5	-
24.	-	-	0.2	1.0	-
25.	-	-	0.2	1.5	-
26.	0.5	-	0.2	-	1000
27.	1.0	-	0.4	-	1000
28.	1.5	-	0.4	-	1000
29.	2.0	-	0.4	-	1000
30.	1.0	0.2	-	-	1000
31.	1.5	0.2	-	-	1000
32.	-	0.2	-	1.5	1000
33.	-	0.2	-	2.0	1000
34. (Liquid)	1.5	0.2	-	-	-

Table 3.3.3 : Plant growth regulator combinations tried for shoot proliferation in *Alstroemeria cv*. Pluto

S. No.	MS + PGR's (mg l^{-1})					
	BAP	IBA	GA_3	Kinetin	TDZ	Activated charcoal
1.	1.0	0.2	0.5	-	-	1000
2.	1.0	0.2	0.5	-	-	-
3.	1.5	0.2	0.5	-	-	1000
4.	1.5	0.2	0.5	-	-	-
5.	2.0	0.4	0.5	-	-	1000
6.	2.0	0.4	0.5	-	-	-
7.	4.0	0.4	1.0	-	-	1000

In-vitro plants were profusely multiplied on Alstroemeria multiplication medium.

3.3.3.1 Observations

1. Per cent proliferation

Based on the number of proliferating cultures.

2. Number of erect shoots

Recorded as number of erect shoots/ rhizome.

3. Number of new rhizome buds

Recorded as rhizome formed with apical buds from cultured rhizome tips.

4. Fresh weight of rhizome/shoot complex (g)

Based on the weight of cluster formed along-with shoots and rhizome per explant cultured.

5. Multiplication index:

Multiplication index was calculated on the basis of number of individual propagules realized from one explant after 6 weeks of culture.

Number of cultures per treatment : 10

Number of replications : 04

Experimental design : Completely Randomized Design

3.3.4 Experiment-IV: Standardisation of callus induction and organogenesis in *Alstroemeria*

Inflorescence buds, shoot nodal segments and leaves of *Alstroemeria cv.* Pluto were used for callus induction and were placed on basal MS solid media consisting of different combinations of Plant growth regulators.

Table 3.3.4 : Plant growth regulator combinations tried for callus induction and organogenesis in *Alstroemeria cv*. Pluto

S. No.	MS + PGR's (mg l^{-1})			
	BAP	**2,4-D**	**NAA**	**GA_3**
1.	0.2	2.0	-	-
2.	0.3	2.5	-	-
3.	0.4	3.0	-	-
4.	0.5	3.5	-	-
5.	0.5	4.0	-	-
6.	0.8	6.0	-	-
7.	1.0	6.5	-	-
8.	0.2	-	2.0	-
9.	0.3	-	2.5	-
10.	0.4	-	3.5	-
11.	0.5	-	4.0	-
12.	0.5	-	4.5	-
13.	0.5	-	5.0	-
14.	-	1.5	1.5	-
15.	-	-	4.0	-
16.	0.5	-	0.4	0.5

3.3.4.1 Observations

1. Callus induction (%)
2. Callus fresh weight (mg)
3. Callus texture/colour
4. Regenerating callus percentage

Number of cultures per treatment : 10

Number of replications : 04

Experimental design : Completely Randomized Design

3.3.5 Experiment V: Standardisation of rooting in *Alstroemeria*

In vitro raised shoots grown on Alstroemeria multiplication medium (solid, solid with activated charcoal and liquid) were cut back to a length of 1 cm and each consisted a rhizome segment with 2-3 buds per shoot and transferred on MS media containing different concentration and combination of auxins.

Table 3.3.5 Plant growth regulator combinations tried for rooting in *Alstroemeria cv.* Pluto

S. No.	MS + PGR's (mg l^{-1})		
	IBA	NAA	Activated charcoal
1.	0.5	-	1000
2.	2.0	-	1000
3.	0.5	-	-
4.	1.0	-	-
5.	2.0	-	-
6.	-	0.5	-
7.	-	1.0	-
8.	-	1.5	-
9.	-	2.0	-
10.	1.0	-	-
11.	1.0	-	1000
12.	1.0	-	-
13.	-	1.5	-
14.	-	2.0	-

3.3.5.1 Observations

1. Days to appearance of root
2. Per cent rooting
3. Number of roots
4. Root length (mm)

Number of cultures per treatment : 10

Number of replications : 04

Experimental design : Completely Randomized Design

3.4 Statistical analysis

Statistical analysis of the data collected for different parameters during the present investigation was subjected to analysis of variance for completely randomized design with four replications (Gomez and Gomez, 1983).

Chapter – 4

EXERIMENTAL RESULTS

The results obtained in the foregoing study are presented in the following sections:

4.1 Experiment–I: Standardisation of disinfection protocol for various explants in *Alstroemeria cv.* Pluto

4.1.1 Culture asepsis

4.1.1.1 Rhizome tips

Data regarding influence of sterilant treatments on culture asepsis per cent of rhizome tip explants after 2 weeks and 4 weeks is presented in Table 4.1.1.1.

At 2 weeks of culture per cent asepsis ranged from 8.24 in explants treated with ethyl alcohol 70% for 30 seconds (S_3) to 79.20 in explants treated with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1 %) dip for 10 minutes and ethyl alcohol 70% for 1 minute.

At 4 weeks highest per cent asepsis (68.08) was recorded in explants treated with Carbendazim 200 ppm (30 minutes) followed by mercuric chloride (0.1 %) dip (10 minutes) and ethyl alcohol 70% for one minute (S_6). This was at par with results recorded in S_8 but significantly superior to all other treatments. Lowest asepsis after 4 weeks was recorded in treatment S_3 (Ethyl alcohol 70% dip for 30 seconds).

4.1.1.2 Rhizome sections

Data pertaining to influence of sterilant treatments on culture asepsis per cent of rhizome section explants of *Alstroemeria cv*. Pluto after 2 weeks and 4 weeks is presented in the Table 4.1.1.2.

Effect of sterilant treatments on culture asepsis per cent of rhizome sections at 2 weeks was highest (63.46) in explants treated with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1 %) dip (10 minutes) and

Table 4.1.1.1: Influence of sterilant treatments on culture asepsis (%) of rhizome tip explants of *Alstroemeria cv*. Pluto

	Sterilants	2 weeks	4 weeks
S_1	Mercuric chloride dip (0.1%) for 5 minutes	27.08 (27.92)	20.84 (24.06)
S_2	Mercuric chloride dip (0.1%) for 10 minutes	41.66 (40.16)	33.33 (35.18)
S_3	Ethyl alcohol (70%) dip for 30 seconds	6.27 (8.24)	4.18 (6.76)
S_4	Ethyl alcohol (70%) dip for 1 minute	37.49 (37.61)	29.16 (32.38)
S_5	Carbendazim 200 ppm for 30 minutes followed by S_1 and S_3	62.49 (52.38)	54.16 (47.43)
S_6	Carbendazim 200 ppm for 30 minutes followed by S_2 and S_4	93.72 (79.20)	85.41 (68.08)
S_7	Carbendazim 400 ppm for 30 minutes followed by S_1 and S_3	81.24 (64.93)	70.83 (57.60)
S_8	Carbendazim 400 ppm for 30 minutes followed by S_2 and S_4	91.65 (75.29)	83.33 (66.25)
	$C.D_{(P\leq0.05)}$	**15.66**	**12.62**

Values in parenthesis are arcsine transformed data in per cent

Table 4.1.1.2: Influence of sterilant treatments on culture asepsis (%) of rhizome section explants of *Alstroemeria cv*. Pluto

	Sterilants	2 weeks	4 weeks
S_1	Mercuric chloride dip (0.1%) for 5 minutes	16.68 (18.12)	12.51 (15.49)
S_2	Mercuric chloride dip (0.1%) for 10 minutes	27.08 (27.92)	22.92 (25.37)
S_3	Ethyl alcohol (70%) dip for 30 seconds	6.27 (8.24)	4.18 (6.76)
S_4	Ethyl alcohol (70%) dip for 1 minute	29.16 (32.63)	27.08 (31.15)
S_5	Carbendazim 200 ppm for 30 minutes followed by S_1 and S_3	62.49 (52.38)	54.16 (47.43)
S_6	Carbendazim 200 ppm for 30 minutes followed by S_2 and S_4	79.16 (63.46)	77.08 (61.63)
S_7	Carbendazim 400 ppm for 30 minutes followed by S_1 and S_3	60.41 (51.06)	52.08 (46.19)
S_8	Carbendazim 400 ppm for 30 minutes followed by S_2 and S_4	74.99 (60.15)	72.91 (58.84)
	$C.D_{(P\leq0.05)}$	**17.00**	**14.86**

Values in parenthesis are arcsine transformed data in per cent

Final treatment with ethyl alcohol (70 %) for one minute. This was followed by 60.15 per cent in explants treated with Carbendazim 400 ppm for 30 seconds, mercuric chloride (0.1 %) dip (10 minutes) and final ethyl alcohol (70 %) for one minute.

At 4 week of culture, difference among the mean per cent asepsis with regard to the effect of sterilant treatments on culture asepsis of rhizome section explant was significant and was highest (61.63 %) in explants treated with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1 %) for (10 minutes) and final treatment with ethyl alcohol (70 %) for one minute. This was followed by 58.84 per cent in explants treated with Carbendazim 400 ppm for 30 seconds, mercuric chloride (0.1 %) dip (10 minutes) and final ethyl alcohol (70 %) for one minute.

Lowest culture asepsis at 2 weeks and 4 weeks of culture was recorded in explants treated with sterilant treatment combination S_3 (Ethyl alcohol 70% dip for 30 seconds).

4.1.1.3 Shoot tip

Influence of sterilant treatments on culture asepsis of shoot tip explants is presented in Table 4.1.1.3.

Highest per cent asepsis at 2 weeks (66.25) and 4 weeks (60.15) was recorded in explants treated with sterilant treatment combination Carbendazim 200 ppm for 15 minutes + mercuric chloride (0.1 %) dip for 3 minutes. This was followed by 63.46 and 54.81 per cent asepsis recorded under sterilant treatment S_4 (Carbendazim 200 ppm for 15 minutes + mercuric chloride dip 0.1 % for 5 minutes) at 2 weeks and 4 weeks of culture respectively. Treatments S_3, S_4, S_5 and S_1 were at par. The lowest culture asepsis per cent of 52.26 was recorded when explants were treated with mercuric chloride 0.1 % dip for 5 minutes (S_2)

Table 4.1.1.3: Influence of sterilant treatments on culture asepsis (%) of shoot tip explants of *Alstroemeria cv*. Pluto

	Sterilants	2 weeks	4 weeks
S_1	Mercuric chloride dip (0.1 %) for 3 minutes	74.99 (60.15)	60.41 (51.06)
S_2	Mercuric chloride dip (0.1 %) for 5 minutes	62.49 (52.26)	52.08 (46.19)
S_3	Carbendazim 200 ppm for 15 minutes followed by S_1	83.33 (66.25)	74.99 (60.15)
S_4	Carbendazim 200 ppm for 15 minutes followed by S_2	79.16 (63.46)	66.66 (54.81)
S_5	Carbendazim 400 ppm for 15 minutes followed by S_1	74.99 (60.15)	62.49 (52.38)
S_6	Carbendazim 400 ppm for 15 minutes followed by S_2	64.58 (53.58)	54.16 (47.43)
	$C.D_{(P\leq 0.05)}$	**7.81**	**7.71**

Values in parenthesis are arcsine transformed data in per cent

4.1.1.4 Shoot nodal segments

Culture asepsis of shoot nodal segments under different sterilant treatments is presented in Table 4.1.1.4.

Per cent asepsis of shoot nodal segments at 2 weeks ranged from 53.58 per cent in explants treated with mercuric chloride 0.1% dip (5 minutes) to 71.95 per cent in explants treated with Carbendazim 200 ppm for 15 minutes followed by mercuric chloride 0.1% dip (3 minutes). At 4 weeks per cent asepsis ranged from 43.79 to 63.46 in explants under the same treatments S_2 and S_3 respectively.

At 2 weeks per cent asepsis recorded under sterilant treatment S_3 was at par with that recorded under S_1 and S_4 but significantly superior to rest of the treatments. However at 4 weeks per cent asepsis under treatments S_3 and S_4 was at par though results obtained with treatment S_3 were significantly superior to all other treatments.

4.1.2 Survival

4.1.2.1 Survival of rhizome sections and rhizome tips

Results pertaining to survival of rhizome sections and rhizome tips after 4 weeks of incubation is presented in the Table 4.1.2.1.

Data revealed highest per cent survival (60.15) of rhizome sections was recorded in explants treated with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride 0.1 % dip (10 minutes) and final ethyl alcohol (70 %) for one minute (S_6).This was significantly superior to survival obtained in all other sterilant treatments. Lowest per cent survival (6.76) was recorded when rhizome sections were treated with ethyl alcohol (70 %) for 30 seconds (S_3).

In rhizome tips highest per cent survival (68.08) was recorded with S_6 sterilant treatment followed by per cent survival (57.60) with S_8 treatment. Lowest per cent survival (14.01) was recorded with S_3.

Table 4.1.1.4 : Influence of sterilant treatments on culture asepsis (%) of shoot nodal segment explants of *Alstroemeria cv*. Pluto

	Sterilants	2 weeks	4 weeks
S_1	Mercuric chloride dip (0.1 %) for 3 minutes	79.16 (63.46)	64.58 (53.58)
S_2	Mercuric chloride dip (0.1 %) for 5 minutes	64.58 (53.58)	47.91 (43.79)
S_3	Carbendazim 200 ppm for 15 minutes followed by S_1	87.48 (71.95)	79.16 (63.46)
S_4	Carbendazim 200 ppm for 15 minutes followed by S_2	79.16 (63.46)	70.83 (57.60)
S_5	Carbendazim 400 ppm for 15 minutes followed by S_1	74.99 (60.15)	66.66 (54.81)
S_6	Carbendazim 400 ppm for 15 minutes followed by S_2	64.58 (53.58)	47.91 (43.79)
	$C.D_{(P\leq0.05)}$	**11.32**	**8.45**

Values in parenthesis are arcsine transformed data in per cent

Table 4.1.2.1: Influence of sterilant treatments on survival (%) of rhizome sections and rhizome tip explants of *Alstroemeria cv.* Pluto

	Sterilants	Rhizome sections	Rhizome tips
S_1	Mercuric chloride dip (0.1%) for 5 minutes	6.27 (8.24)	14.59 (16.81)
S_2	Mercuric chloride dip (0.1%) for 10 minutes	16.68 (18.12)	22.91 (28.36)
S_3	Ethyl alcohol (70%) dip for 30 seconds	4.18 (6.76)	10.43 (14.01)
S_4	Ethyl alcohol (70%) dip for 1 minute	24.99 (29.83)	31.24 (33.86)
S_5	Carbendazim 200 ppm for 30 minutes followed by S_1 and S_3	49.99 (44.99)	62.49 (52.38)
S_6	Carbendazim 200 ppm for 30 minutes followed by S_2 and S_4	74.99 (60.15)	85.41 (68.08)
S_7	Carbendazim 400 ppm for 30 minutes followed by S_1 and S_3	49.99 (44.99)	58.33 (49.83)
S_8	Carbendazim 400 ppm for 30 minutes followed by S_2 and S_4	68.74 (56.13)	70.83 (57.60)
	$C.D_{(P\leq0.05)}$	**14.87**	**14.31**

Values in parenthesis are arcsine transformed data in per cent

*Data recorded at the end of 4 weeks of culture

4.1.2.2 Survival of shoot tips and shoot nodal segments

There were significant differences in shoot tips and shoot nodal segment explants survival in response to sterilant treatments. Results regarding shoot tip and shoot nodal segments are presented in Table 4.1.2.2.

Highest per cent survival of shoot tips (56.13) was recorded in explants treated with Carbendazim 200 ppm for 15 minutes followed by mercuric chloride (0.1 %) dip for 3 minutes. This was significantly superior to treatments were Carbendazim strength was 400 ppm with 3 or 5 minute mercuric chloride (0.1 %) dip. Lowest per cent survival (29.83) was recorded in explants treated with mercuric chloride (0.1 %) dip for 5 minutes.

In case of shoot nodal segments highest per cent survival of (52.38) recorded with Carbendazim 200 ppm for 15 minutes followed by mercuric chloride (0.1 %) dip for 3 minutes (S_3) which was at par with treatment were Carbendazim concentration was increased to 400 ppm with same mercuric chloride (0.1 %) exposure duration (S_5) or were Carbendazim concentration was left unchanged but followed by 5 minute mercuric chloride (0.1 %) dip (S_6). Survival per cent in all the above treatments was significantly superior to rest of the treatments. Lowest per cent survival of (35.18) was recorded in explants treated with mercuric chloride (0.1 %) dip for 5 minutes.

4.2 Experiment II: Standardization of media and growth regulator combination for culture establishment in *Alstroemeria cv.* Pluto.

4.2.1 Per cent establishment

In all 25 plant growth regulator combinations in MS-media were tried to evaluate the influence on culture establishment of rhizome tips and rhizome sections. Results are presented in Table 4.2.1.

Perusal of data reveal significant differences among various treatments in terms of culture establishment. In case of rhizome tips, highest per cent

Table-4.1.2.2 : Influence of sterilant treatments on survival (%) of shoot tips and shoot nodal segment explants of *Alstroemeria cv.* Pluto

	Sterilants	Shoot tips	Shoot nodal segments
S_1	Mercuric chloride dip (0.1 %) for 3 minutes	41.66 (40.16)	49.99 (45.00)
S_2	Mercuric chloride dip (0.1 %) for 5 minutes	24.99 (29.83)	33.33 (35.18)
S_3	Carbendazim 200 ppm for 15 minutes followed by S_1	68.74 (56.13)	62.49 (52.38)
S_4	Carbendazim 200 ppm for 15 minutes followed by S_2	47.91 (43.79)	54.16 (47.43)
S_5	Carbendazim 400 ppm for 15 minutes followed by S_1	54.16 (47.43)	58.33 (49.83)
S_6	Carbendazim 400 ppm for 15 minutes followed by S_2	45.83 (42.56)	52.08 (46.19)
	$C.D_{(P\leq 0.05)}$	**7.66**	**8.17**

Values in parenthesis are arcsine transformed data in per cent

*Data recorded at the end of 4 weeks of culture

Table 4.2.1 : Influence of growth regulator combinations on culture establishment (%) in *Alstroemeria cv.* Pluto

Treatment	MS + PGRs mg l^{-1}					Rhizome tips*	Rhizome sections*
	BAP	IBA	NAA	Kinetin	Activated charcoal		
T_1	0.2	0.02	-	-	-	35.41 (36.49)	25.75 (30.48)
T_2	0.4	0.02	-	-	-	35.83 (36.74)	29.91 (33.11)
T_3	0.6	0.02	-	-	-	41.66 (40.165)	35.49 (36.46)
T_4	0.8	0.02	-	-	-	86.66 (69.02)	37.49 (37.61)
T_5	1	0.02	-	-	-	86.88 (68.83)	38.91 (38.54)
T_6	1	0.2	-	-	-	88.92 (70.84)	39.74 (39.06)
T_7	1.5	0.2	-	-	-	70.83 (57.60)	37.49 (37.73)
T_8	1.5	0.4	-	-	-	41.35 (39.95)	29.16 (32.63)
T_9	1.5	0.6	-	-	-	38.12 (38.04)	22.91 (28.52)
T_{10}	2	0.2	-	-	-	41.35 (39.95)	22.16 (28.01)
T_{11}	2	0.4	-	-	-	33.08 (35.11)	20.33 (26.71)
T_{12}	2	0.6	-	-	-	31.00 (33.79)	18.74 (25.56)
T_{13}	2.5	0.2	-	-	-	39.58 (38.93)	20.33 (26.71)
T_{14}	2.5	0.4	-	-	-	33.08 (35.11)	18.74 (25.56)
T_{15}	2.5	0.6	-	-	-	31.00 (33.79)	16.66 (24.09)
T_{16}	-	0.2	-	0.5	-	30.00 (33.17)	16.66 (24.09)
T_{17}	-	-	0.2	1	-	30.11 (33.24)	18.74 (25.56)
T_{18}	0.5	-	0.2	-	1000	31.00 (33.79)	18.75 (22.48)
T_{19}	1	-	0.4	-	1000	29.00 (32.53)	16.66 (24.09)
T_{20}	2	-	0.4	-	1000	27.93 (31.87)	16.66 (24.09)
T_{21}	1	0.2	-	-	1000	94.55 (78.25)	41.74 (40.24)
T_{22}	1.5	0.2	-	-	1000	93.74 (77.43)	39.58 (38.93)
T_{23}	-	0.2	-	1.5	1000	39.58 (38.93)	39.58 (38.93)
T_{24}	-	0.2	-	2	1000	29.00 (32.53)	37.49 (37.73)
T_{25} (Liquid media)	1.5	0.2	-	-	-	99.99 (89.42)	68.74 (56.13)
	$C.D_{(P\leq0.05)}$					**6.37**	**6.32**

Values in parenthesis are arcsine transformed data in per cent

*Data recorded at the end of 6 weeks of culture

establishment (89.42) was recorded under the plant growth regulator combination BAP + IBA: 1.5 + 0.2 mg l^{-1} in MS- liquid media (Plate 1a).

Highest establishment of rhizome tips on solid media (78.25 %) was recorded under T_{21} (BAP + IBA + Activated charcoal: 1.0 + 0.2 + 1000 mg l^{-1}) which was at par with T_{22} (BAP + IBA + Activated charcoal: 1.5 + 0.2 + 1000 mg l^{-1}). Establishment in T_{21} was significantly superior than that recorded under T_6 were similar concentration of BAP and IBA except for activated charcoal were employed (Plate 1b and 1c). Lowest establishment per cent (31.87) was recorded under T_{20} (BAP + NAA + Activated charcoal: 2.0 + 0.4 + 1000 mg l^{-1}).

Establishment in rhizome sections was relatively lower in comparison to rhizome tips. Highest establishment (56.13 %) was recorded in liquid media (T_{25}) which was significantly superior than average establishment under rest of the plant growth regulator combinations (Plate 1d). Establishment of rhizome sections in solid media with activated charcoal was significantly superior than that recorded in solid media without activated charcoal (Plate 1e and 1f). Per cent establishment under T_{21} (BAP + IBA + Activated charcoal: 1.0 + 0.2 + 1000 mg l^{-1}) was 40.24 which was significantly superior than 39.09 and 37.73 recorded under T_7 and T_6 (BAP + IBA: 1.5 + 0.2 mg l^{-1} and BAP + IBA : 1.0 + 0.2 mg l^{-1} respectively). Lowest establishment per cent (22.48) in rhizome sections was recorded under T_{18} (BAP + NAA + activated charcoal: 0.5 + 0.2 + 1000 mg l^{-1}).

4.2.2 Days to sprouting and number of sprouted buds

Data regarding influence of plant growth regulator combinations on days to sprouting and number of sprouted buds in rhizome tips and rhizome sections is presented in Table 4.2.2.

Liquid media significantly improved days to sprouting and number of sprouted buds in comparison to solid media. Least number of days to sprouting (9.18 and 9.98 in rhizome tips and rhizome sections respectively) and highest mean number of sprouted buds (4.05 and 3.00 in rhizome tips and rhizome

Table 4.2.2 : Influence of growth regulator combinations on days to sprouting and number of sprouted buds in *Alstroemeria cv.* Pluto

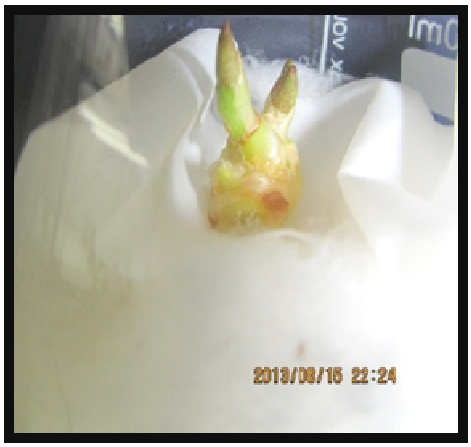
Treatment	MS + PGRs mg l^{-1}					Days to sprouting		No. of sprouted buds*	
	BAP	IBA	NAA	Kinetin	Activated charcoal	Rhizome tips	Rhizome sections	Rhizome tips	Rhizome sections
T_1	0.4	0.02	-	-	-	5.32	5.87	0.87	0.62
T_2	0.6	0.02	-	-	-	8.07	8.12	1.00	0.75
T_3	0.8	0.02	-	-	-	11.52	11.71	1.00	0.98
T_4	1	0.02	-	-	-	14.56	14.63	1.21	1.00
T_5	1	0.2	-	-	-	15.06	14.13	1.97	1.91
T_6	1.5	0.4	-	-	-	15.45	15.76	0.92	0.75
T_7	1.5	0.6	-	-	-	16.23	16.47	0.82	0.62
T_8	2	0.2	-	-	-	10.23	11.67	1.00	0.80
T_9	2	0.4	-	-	-	12.13	12.27	0.93	0.75
T_{10}	2	0.6	-	-	-	12.38	13.23	0.71	0.62
T_{11}	2.5	0.2	-	-	-	14.06	14.25	0.77	0.72
T_{12}	2.5	0.6	-	-	-	14.07	14.31	0.70	0.62
T_{13}	-	0.2	-	0.5	-	12.10	12.87	0.92	0.87
T_{14}	-	-	0.2	1	-	11.03	11.71	0.87	0.56
T_{15}	0.5	-	0.2	-	1000	10.72	11.67	1.20	1.00
T_{16}	1	-	0.4	-	1000	11.03	11.98	0.91	0.87
T_{17}	2	-	0.4	-	1000	11.61	12.16	0.75	0.62
T_{18}	1	0.2	-	-	1000	11.08	11.91	2.25	2.05
T_{19}	1.5	0.2	-	-	1000	12.50	12.92	2.10	1.96
T_{20}	-	0.2	-	1.5	1000	13.13	13.81	0.93	0.87
T_{21}	-	0.2	-	2	1000	13.52	14.00	0.80	0.62
T_{22} (Liquid)	1.5	0.2	-	-	-	9.18	9.98	4.05	3.00
C.D $_{(P\leq0.05)}$						**0.63**	**2.03**	**0.54**	**0.56**

*Data recorded at the end of 6 weeks of culture

Establishment of rhizome tips in liquid media

1a) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Establishment of rhizome sections in liquid media



1d) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Establishment of rhizome tips on transparent solid media

1b) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Establishment of rhizome sections on transparent solid media

1e) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Establishment of rhizome tips on activated charcoal media

1c) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Establishment of rhizome sections on activated charcoal media

1f) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Plate-1 : Culture establishment

sections respectively) were recorded in liquid media supplemented with BAP and IBA: 1.5 + 0.2 mg l^{-1} (Plate 2a and 2d). The results are comparable with those recorded on solid media supplemented with BAP + IBA + Activated charcoal : 1.0 + 0.2 + 1000 mg l^{-1} (11.08 and 11.91 days recorded to sprouting in rhizome tips and rhizome sections; 5.25 and 2.25 number of sprouted buds recorded in rhizome tips and rhizome sections).Number of sprouted buds was significantly higher on solid media with activated charcoal as compared to solid media without activated charcoal irrespective of having same growth regulator regime (Plate 2b, 2c, 2e and 2f).

Highest days to sprouting (16.23 and 16.47 in rhizome tips and rhizome sections respectively) was recorded under T_7 ((BAP + IBA: 1.5 + 0.6 mg l^{-1}). Lowest number of sprouted buds 0.62 in rhizome tips was recorded in T_6, T_7, T_8, T_{11} and T_{13}. Lowest number of sprouted buds (0.70) in case of rhizome sections was recorded under T_{12}.

4.2.3 Influence of other plant growth regulator combinations on culture establishment

In addition to data presented in Table 4.2.1, several other plant growth regulator combinations were tried (Appendix–I). However, both the explant types (rhizome tips and rhizome sections) failed to establish in the below mentioned plant growth regulator combinations presented in Appendix–I.

4.3 Experiment-III : Standardisation of callus induction and organogenesis in *Alstroemeria* cv. Pluto

4.3.1 Per cent callus induction, callus fresh weight, callus type

Data regarding the per cent callus induction, callus texture/colour and callus fresh weight under different plant growth regulator combinations in inflorescence bud explant of *Alstroemeria cv.* Pluto is presented in Table 4.3.1.

In inflorescence buds of *Alstroemeria cv.* Pluto differences in means recorded for per cent callus induction, callus fresh weight under different plant growth regulator concentrations were significant. Inflorescence bud explant in

Table 4.3.1: Influence of growth regulator combinations on callusing in inflorescence bud explant of *Alstroemeria cv.* Pluto

MS + PGRs mg l^{-1}			Callus induction (%)	Callus fresh weight (mg)*	Callus Texture/colour
Treatment	**BAP**	**NAA**			
T_1	0.2	2	62.49 (52.26)	1.57	Friable white green (FWG)
T_2	0.4	3.5	18.75 (19.21)	0.82	FWG (Friable white green)
T_3	0.5	4	93.73 (79.37)	2.98	FWG (Friable white green)
T_4	0.5	4.5	35.41 (36.49)	1.05	FWG (Friable white green)
C.D $_{(P\leq 0.05)}$			**19.06**	**0.50**	

Values in parenthesis are arcsine transformed data in per cent

*Data recorded at the end of 6 weeks of culture

Sprouted buds of rhizome tips in liquid media

2a) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Sprouted buds of rhizome sections in liquid media

2d) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Sprouted buds of rhizome tips in transparent solid media

2b) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Sprouted buds of rhizome sections in transparent solid media

2e) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Sprouted buds of rhizome tips in activated charcoal media

2c) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Sprouted buds of rhizome sections in activated charcoal media

2f) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Plate-2 : Sprouted buds

MS-medium fortified with BAP + NAA : 0.5 + 4.0 mg l^{-1} revealed highest per cent callus induction (79.37) and the lowest (19.21) was recorded under BAP + NAA: 0.4 + 3.5 mg l^{-1} which was statistically at par with a treatment T_4 (BAP + NAA : 0.5 + 4.5 mg l^{-1}).

Highest mean callus fresh weight (2.98 mg) under NAA + BAP: 4 + 0.5 mg l^{-1} was significantly superior to all other NAA based growth regulator combinations. This was followed by (1.57 mg) under NAA + BAP: 2.0 + 0.2 mg l^{-1}. Lowest callus fresh weight (0.82 mg) was recorded with NAA +BAP: 3.5 + 0.4 mg l^{-1}.

Friable white green callus was observed in calluses growing in media supplemented with NAA.

4.3.2 Callusing in inflorescence buds

In addition to data presented in Table 4.3.1 several other plant growth regulator treatment combinations were also tried (Appendix–II). However, inflorescence buds failed to develop any callus on the said plant growth regulator combinations.

4.1.4.3 Callusing in leaves and shoot nodal segments.

Plant growth regulator combinations on MS- media tried for callus induction in *Alstroemeria cv.* Pluto leaf explants are presented in Appendix–III. However, no response in terms of callus induction was obtained.

Further plant growth regulator combinations tried for regeneration of callus presented in Appendix–IV, failed to elicit response in terms of regeneration from callus.

4.4 Experiment IV: Standardisation of shoot proliferation in *Alstroemeria cv.* Pluto

4.4.1 Per cent establishment, number of erect shoots, number of new rhizome buds, rhizome fresh weight/shoot complex and multiplication index

Data pertaining to effect of various plant growth regulator combinations on rhizome tip proliferation (%), number of erect shoots and number of new rhizome buds in *Alstroemeria cv.* Pluto are presented in Table 4.4.1.

Proliferation of rhizome tip explants in media fortified with activated charcoal was significantly higher as against the treatments were activated charcoal was not used. Highest per cent proliferation (88.85) was recorded with treatment T_1 (BAP + IBA + GA_3 + activated charcoal: 1.0 + 0.2 + 0.5 + 1000 mg l^{-1}), T_3 (BAP + IBA + GA_3 + activated charcoal: 1.5 + 0.2 + 0.5 + 1000 mg l^{-1}), and T_5 (BAP + IBA + GA_3 + activated charcoal: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}).Per cent proliferation recorded under all the above treatments was significantly higher than that recorded under T_2 (BAP + IBA + GA_3: 1.0 + 0.2 + 0.5 mg l^{-1}), T_4 (BAP + IBA + GA_3: 1.5 + 0.2 + 0.5 mg l^{-1}) and T_6 (BAP + IBA + GA_3: 2.0 + 0.4 + 0.5 mg l^{-1}) (Plate 3a, 3b, 3c). Lowest per cent proliferation (26.66) was recorded with T_7 (BAP + IBA + GA_3 + activated charcoal: 4.0 + 0.4 + 1.0 + 1000 mg l^{-1}).

Results indicate that explants placed in activated charcoal fortified medium on an average yielded significantly higher number of erect shoots in comparison to explants on media without activated charcoal. Highest number of erect shoots (5.75/explant) was recorded in MS- media supplemented with BAP + IBA + GA_3 + Activated charcoal: 2.0 +0.4 + 0.5 + 1000 mg l^{-1} (Plate 3e). This was statistically at par with 5.00 erect shoots recorded in T_1 (BAP + IBA + GA_3 + Activated charcoal: 1.0 +0.2 + 0.5 + 1000 mg l^{-1}) (Plate 3f). Number of shoots recorded in media not supplemented with activated charcoal, T_2 (BAP + IBA + GA_3 : 1.0 +0.2 + 0.5 mg l^{-1}), T_4 (BAP + IBA + GA_3 : 1.5 +0.2 + 0.5 mg l^{-1}), T_6 (BAP + IBA + GA_3 : 2.0 +0.4 + 0.5 mg l^{-1}) was 3.25, 2.75 and 3.00 respectively (Plate 3d). The values for T_2, T_4 and T_6 were statistically at par. Lowest number of erect shoots (1.50) was recorded in T_7 (BAP + IBA + GA_3 + Activated charcoal: 4.0 +0.4 + 1.0 + 1000 mg l^{-1}).

Table 4.4.1 : Influence of growth regulator combinations on proliferation in rhizome tip explants of *Alstroemeria cv.* Pluto

Treatment	MS + PGRs mg l^{-1}				Per cent proliferation	No. of erect shoots*	No. of new rhizome buds**	Rhizome fresh weight/shoot complex (g)	Multiplication index/cycle
	BAP	IBA	GA_3	Activated charcoal					
T_1	1	0.2	0.5	1000	99.96 (88.85)	5.00	3.50	5.77	× 2.52
T_2	1	0.2	0.5	-	85.83 (68.31)	3.25	2.00	4.75	× 1.67
T_3	1.5	0.2	0.5	1000	99.96 (88.85)	4.25	2.25	5.05	× 1.95
T_4	1.5	0.2	0.5	-	66.66 (54.81)	2.75	1.12	4.06	× 0.95
T_5	2	0.4	0.5	1000	99.96 (88.85)	5.75	3.75	6.05	× 2.76
T_6	2	0.4	0.5	-	81.66 (65.01)	3.00	1.75	4.45	× 1.50
T_7	4	0.4	1	1000	31.26 (26.66)	1.50	0.62	3.55	× 0.30
$C.D_{(P \leq 0.05)}$					**17.23**	**1.55**	**1.30**	**0.37**	**0.43**

Values in parenthesis are arcsine transformed data in per cent
*Data was recorded after 6 weeks of culture
**Data was recorded after 6 weeks of culture.

Rhizome tip proliferation on transparent solid media

3a) BAP + IBA + GA_3: 1.0 + 0.2 + 0.5 mg l^{-1}

Erect shoots on transparent solid media

3d) BAP + IBA + GA_3: 1.0 + 0.2 +0.5 mg l^{-1}

Rhizome tip proliferation in media with activated charcoal

3b) BAP + IBA + GA_3 + AC: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}

Erect shoots in media with activated charcoal

3e) BAP + IBA + GA_3 + AC: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}

Rhizome tip proliferation in media with activated charcoal

3c) BAP + IBA + GA_3 + AC: 1.0 + 0.2 + 0.5 + 1000 mg l^{-1}

Erect shoots in media with activated charcoal

3f) BAP + IBA + GA_3 + AC: 1.0 + 0.2 + 0.5 + 1000 mg l^{-1}

Plate-3 : Proliferation

Number of new rhizome buds on explants also followed the same pattern with significantly higher number of new rhizome buds recorded under T_5 (3.75) followed by (3.50) buds under T_1. Lowest number of new rhizome buds (0.62) was recorded under T_7 (BAP + IBA + GA_3 + Activated charcoal: 4.0 +0.4 + 1.0 + 1000 mg l^{-1}).

Rhizome fresh weight/shoot complex in media fortified with Activated charcoal was significantly higher as compared to treatments were activated charcoal was not used. Highest rhizome fresh weight/shoot complex (6.05 g) was recorded with treatment (T_5). This was followed by 5.77 g under T_1 and 5.05 g under T_3. Lowest rhizome fresh weight/shoot complex (3.55 g) was recorded under T_7.

Results indicate that explants placed in activated charcoal fortified medium on an average exhibited significantly higher multiplication index in comparison to explants in media without activated charcoal. Highest multiplication index of (2.76) was recorded in MS- media supplemented with (BAP + IBA + GA_3 + Activated charcoal: 2.0 +0.4 + 0.5 + 1000 mg l^{-1}).This was statistically at par with a multiplication index of (2.52) recorded under T_1 and multiplication index of (1.95) under T_3.

Multiplication indices recorded in media not supplemented with activated charcoal T_2 (BAP + IBA + GA_3: 1.0 + 0.2 + 0.5 mg l^{-1}), T_4 (BAP + IBA + GA_3: 1.5 + 0.2 + 0.5 mg l^{-1}) and T_6 (BAP + IBA + GA_3: 2.0 + 0.4 + 0.5 mg l^{-1}) was 1.67, 0.95 and 1.50 respectively. Mean multiplication index values recorded under T_2, T_4 and T_6 were statistically at par. Lowest multiplication index (0.30) was recorded under treatment T_7 (BAP + IBA + GA_3 + Activated charcoal: 4.0 +0.4 + 1.0 + 1000 mg l^{-1}).

In addition to plant growth regulator combinations presented in Table 4.4.1, other combinations were no response in terms of proliferation was achieved are presented in Appendix – V.

4.5 Experiment V: Standardisation of rooting in *Alstroemeria*

4.5.1 Per cent rooting and Days to appearance of root

Data pertaining to the influence of plant growth regulators on per cent rooting and days to appearance of root is presented in the Table 4.5.1.

Results indicate that explants incubated on MS-liquid media supplemented with NAA 1.5 mg l^{-1} resulted in significantly higher per cent rooting (54.81) (Plate 4a).This was statistically at par with per cent rooting (48.63 and 44.99) recorded under the treatments (T_{10} and T_8) in liquid based media supplemented with NAA 2.0 mg l^{-1} and IBA 1.0 mg l^{-1} respectively.

In case of explants incubated on solid media, highest per cent rooting (42.56) was recorded under NAA 1.5 mg l^{-1}. This was statistically at par with per cent rooting under T_9. Also results indicate that explants placed in activated charcoal supplemented media recorded least per cent rooting (30.00). Hence presence of Activated charcoal in rooting media fortified with auxin revealed non-significant effect on per cent rooting of rhizome explants.

Perusal of data reveal non-significant effect of plant growth regulators on differences in mean number of days to appearance of root. Lowest days to appearance of root (10.87) was recorded in MS-liquid media fortified with NAA 1.5 mg l^{-1}. This was statistically at par with other treatments.

Explants incubated on solid media supplemented with IBA 1.0 and 2.0 mg l^{-1} recorded 12.75 and 13.12 days to appearance of root respectively. Highest number of days to root appearance (15.37) was recorded in explants on MS solid medium supplemented with IBA 2.0 mg l^{-1} and activated charcoal 1000 mg l^{-1}.

Table 4.5.1 : Influence of growth regulator combinations on rooting in rhizome explants in *Alstroemeria cv.* Pluto

Treatment	MS + PGRs mg l^{-1}			Rooting (%)	Days to appearance of root
	IBA	NAA	Activated charcoal		
T_1	2	-	1000	25.00 (30.00)	15.37
T_2	0.5	-	-	25.00 (30.00)	12.87
T_3	1	-	-	27.08 (31.15)	12.75
T_4	2	-	-	25.00 (26.55)	13.12
T_5	-	1	-	37.49 (37.73)	12.62
T_6	-	1.5	-	45.83 (42.56)	12.45
T_7	-	2	-	39.58 (38.93)	13.75
T_8 (Liquid)	1	-	-	49.99 (44.99)	11.04
T_9 (Liquid)	-	1.5	-	66.66 (54.81)	10.87
T_{10} (Liquid)	-	2	-	56.24 (48.63)	12.50
	C.D $_{(P\leq0.05)}$			**10.49**	**NS**

Values in parenthesis are arcsine transformed data in per cent

NS = Non significant

4a) Rooting in liquid media (NAA 1.5 mg l^{-1})

4b) Number of roots (NAA 1.5 mg l^{-1})

4c) Number of roots (NAA 1.5 mg l^{-1})

Plate-4 : Rooting

4.5.2 Number of roots and root length

Data pertaining to the effect of various auxins on number of roots and root length (mm) in *Alstroemeria cv.* Pluto is presented in the Table 4.5.2.

Data reveal significantly higher root number in explants incubated on liquid media supplemented with NAA (Plate 4b and 4c). Root number in explants under T9 and T10 (Liquid MS media supplemented with 1.5 or 2.0 mg NAA l^{-1}) was 3.12 and 2.81 respectively.

Lowest root number (1.12) was recorded in explants on MS solid media supplemented with IBA + Activated charcoal: 2.0 + 1000 mg l^{-1}.

In addition to plant growth regulators presented in Table 4.5.1, other combinations were no response in terms of per cent rooting was achieved are presented in Appendix–VI .

Table 4.5.2 : **Influence of growth regulator combinations on root number and length in rhizome tip explants in *Alstroemeria cv.* Pluto**

	MS + PGRs mg l^{-1}				
Treatment	**IBA**	**NAA**	**Activated charcoal**	**Number of roots**	**Root length (mm)**
T_1	2	-	1000	1.12	12.12
T_2	0.5	-	-	1.25	12.31
T_3	1	-	-	2.00	14.65
T_4	2	-	-	1.87	12.75
T_5	-	1	-	1.37	14.90
T_6	-	1.5	-	2.50	15.50
T_7	-	2	-	2.12	14.11
T_8 (Liquid)	1	-	-	2.11	15.12
T_9 (Liquid)	-	1.5	-	3.12	16.42
T_{10} (Liquid)	-	2	-	2.81	15.01
			C.D $_{(P \leq 0.05)}$	**1.33**	**NS**

NS= Non-significant

Chapter – 5

DISCUSSION

In light of the available literature and data generated in the foregoing study, the results are discussed in the following sections.

5.1 Experiment–I : Standardisation of disinfection protocol for various explants in *Alstroemeria cv.* Pluto

5.1.1 Surface sterilization, culture asepsis and survival

5.1.1.1 Culture asepsis and survival of rhizome tips, rhizome sections, shoot tips and shoot nodal segments

Various surface sterilization strategies have been devised in *Alstroemeria* by different workers according to varied morphology of the explants in order to achieve desirable level of culture asepsis and survival.

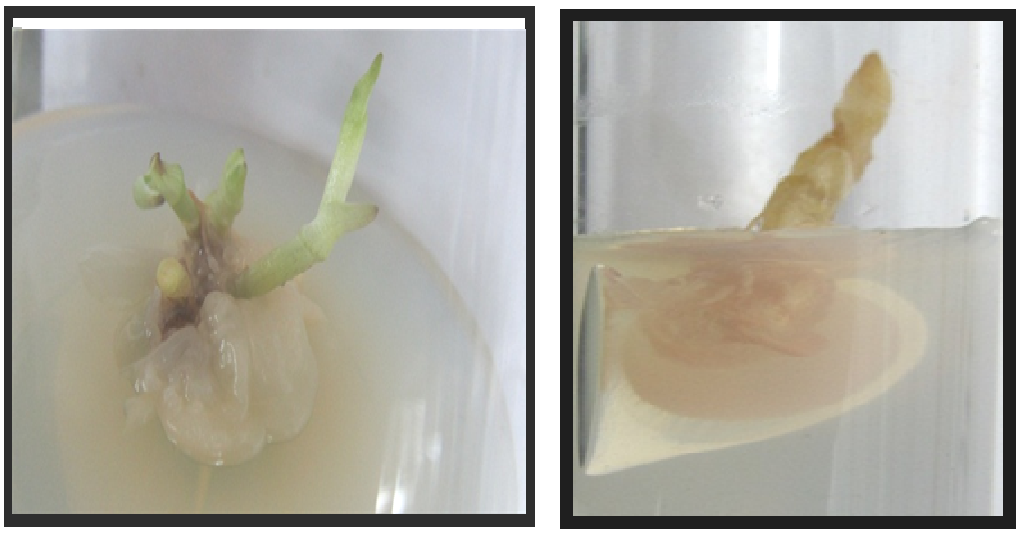
In *Alstroemeria* rhizome is the main explant an underground structure and carries a significant microbial load. Therefore, achieving culture asepsis and subsequent survival of explants is a significant challenge in *Alstroemeria.*

In the current study, rhizome tips and rhizome sections were surface sterilized for 5.0 and 10 minutes in 0.1% mercuric chloride after a 30 minute surfactant treatment fortified with 200 and 400 ppm Carbendazim and final treatment with ethyl alcohol (70 %) for one minute. Significant differences among means in terms of per cent asepsis and subsequent survival were observed for both explants and sterilant combinations in rhizome tips and rhizome sections (Tables 4.1.1.1, 4.1.1.2 and 4.1.2.1). Highest culture asepsis was achieved in rhizome tips. Maximum asepsis in rhizome tips may be attributed to its lesser surface area exposed as a result of single cut at the distal end of the rhizome and hence less area for entry of potential infections. This is in comparison to rhizome sections where two cuts are made at the proximal and distal ends and hence more exposed tissue. Further rhizome tips are compact structures with relatively fewer subtending organs like roots and tubercles and hence have less quantity of

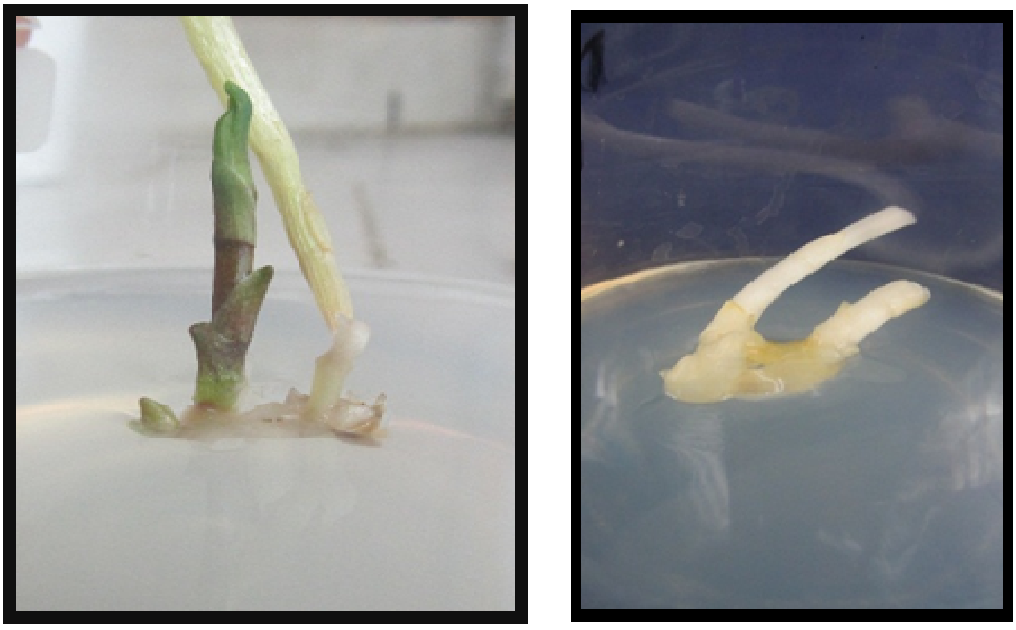
extraneous material like dirt, soil etc adhering which may have contributed to higher asepsis in comparison to rhizome sections.

Among the sterilant combinations, a 10 minute mercuric chloride (0.1 %) solution dip significantly improved culture asepsis in comparison to 5 minute dip. Ethyl alcohol 70 % treatment alone for one minute proved better (S_4) than a 30 seconds treatment with ethyl alcohol 70 %(S_3).However one minute ethyl alcohol treatment at the end of sterilization procedure involving Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1%) for 10 minutes under laminar flow hood significantly increased culture asepsis (79.20%) (Plate 5b). The results indicate passing explants through fungicidal treatment followed by mercuric chloride and final treatment with ethyl alcohol is indispensable for culture asepsis in rhizome explants of *Alstroemeria*. Concentrated ethanol is a powerful disinfectant that kills majority of fungal and bacterial spores through instant dehydration. However increasing concentration of Carbendazim from 200 to 400 ppm did not improve the final level of culture asepsis any further. The difference between the two sets of treatments involving 5.0 and 10 minute mercuric chloride dip and 30 seconds and one minute ethyl alcohol (70 %) treatment underlines the importance of minimally adequate time duration of final mercuric chloride (0.1 %) for 10 minutes and ethyl alcohol (70 %) for one minute surface sterilization procedure under the laminar flow hood.

Overall results indicate that rhizome tips survived better than the rhizome sections which may be due to less injured area in the former and hence lesser quantity of exudates. In the current experiment elusion of exudates from explants was a major cause of physiological mortality *in vitro* (Plate 5a). Data reveal the importance of ethyl alcohol in the eventual survival of the aseptic explants recorded at the end of 4 weeks of culture. Use of carbendazim followed by ethyl alcohol dip significantly improved culture survival both in rhizome sections and rhizome tips. Whereas the former decreased mortality because of fungal infections ethyl alcohol may have contributed in survival by mitigating the harmful effects



5a) Exudate production from explants



5b) Aseptic cultures

of mercuric chloride. Ethyl alcohol being a dehydrant may have played a role in drawing out excess Hg^{2+} ions out of the explant tissues along with the exudates which may have significantly contributed to higher survival in explants under S_6 and S_8 sterilant treatment. Mohanty *et al*. (2005) advocated use of final wash in 1% hypertonic KCl solution to draw out any traces of Hg^{2+} from the explant tissue.

Carbendazim, mercuric chloride and ethyl alcohol have been used by several workers to achieve the desired level of culture asepsis and survival *in vitro*. Hakkart and Versluijs (1985) initially rinsed the rhizomes of *Alstroemeria cv's* Rosario, Toledo, and Jubilee with tap water and later dipped for few seconds in 70% ethyl alcohol. Final sterilization was done in a laminar flow hood with 5 % Ca Hypochlorite (CaOCl) followed by rinsing 3 times with sterile water. Pierik *et al*. (1988) reported use of 70 % ethyl alcohol for 2-3 seconds followed by 20 minute dip in 1.5 % NaOCl (with few drops of Tween 20). Jyothi *et al*. (2008) reported use of 1.0 % mercuric chloride for 8 minutes in ginger a plant with an underground rhizome similar in architecture as *Alstroemeria.* Sathyagowri and Seran (2011) also reported reasonable level of culture asepsis and survival with Carbendazim (0.3%) + Doxycycline (0.2%) for 10 minutes followed by 70% ethanol for 1 minute in ginger rhizome explants with buds. In the present study 10 minute dip in mercuric chloride (0.1 %) appears to be appropriate for a reasonable level of culture asepsis and to avoid mortality of explants.

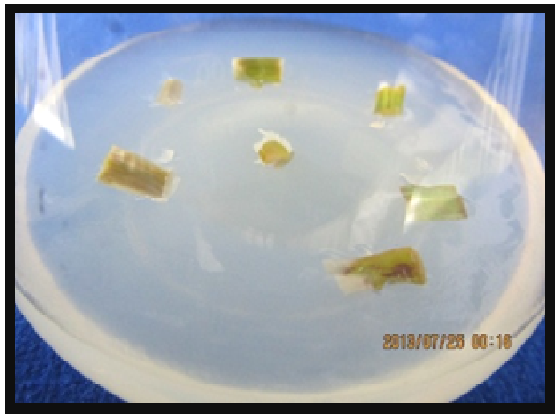
In the current study, shoot tips and shoot nodal segments were surface sterilized for 3 and 5 minute in 0.1% mercuric chloride after a 15 minute surfactant treatment fortified with 200 and 400 ppm Carbendazim. Significant differences among means were observed for both explants. Highest culture asepsis was achieved in shoot nodal segments rather than shoot tips. Lower asepsis of shoot tips may be due to bud morphology that allows accumulation of extraneous material among loosely placed leaves which are also sticky. However, survival was relatively better in shoot tips than shoot nodal segments. There was less

elusion of exudates from shoot tips in comparison to shoot nodal segments. Less exudates in shoot tips may be due to more proximal position in shoots as most of the exudates flow down and accumulate in the lower portion of flowering shoots which translated into more exudate outflow *in vitro* in the latter and hence less survival. Data also point to the importance of use of carbendazim at 200 and 400 ppm in improving asepsis and survival of the explants. Shoot tips and shoot nodal segments are tender structures and hence use of ethyl alcohol is not advocated. Seyyedyousefi *et al*. (2013) also advocated fragments of stem containing node and internode were washed thoroughly under running tap water for 20 minutes and disinfected

5.2 Experiment-II : Standardisation of callus induction and organogenesis in *Alstroemeria*

5.2.1 Callusing

In the current study leaves, inflorescence buds and shoot nodal segments were employed to generate callus. However, leaves and nodal segments failed to generate any callus during the exploratory trials and hence were not included as explant in the final experiment. The main stumbling block in callus generation from leaves was due to chlorophyll loss during the exploratory trial and may be attributed to its thin leaf architecture (Plate 6a). Khaleghi (2011) also reported poor response of leaf explants of Alstroemeria in terms of callus development. In contrast to our studies Seyyedyousefi *et al*. (2013) reported success in case of nodal and internodal segment explants in terms of callus development. Also nodal explants were used for the production of a high level of compact callus (Kim *et al*., 2001). Sixteen growth regulator combinations involving 2, 4-D and NAA in combination with cytokinin BAP and Gibberelic acid were used in the standardisation experiments. All these 16 plant growth regulator combinations (Appendix-III) failed to elicit any response in terms of callus induction in case of leaf and shoot nodal segment explants and also out of these 16 plant growth regulator combinations 12 growth regulator combinations (Appendix-II) failed



6a) Chlorophyll loss in leaves on callus media

6b) Flower development on callus media

6c) Callus induction in inflorescence buds (BAP + NAA: 0.5 + 4.0 mg l^{-1})

Plate-6 : Callusing

callusing in inflorescence buds. Besides the callus induction, flower development was recorded in inflorescence bud explants which may be due to already differentiated flowers in the buds that sprouted out once placed on favourable culture media (Plate 6b).

Callusing in inflorescence bud explants was recorded in terms of callus induction (%), callus fresh weight explant^{-1} and callus type. Perusal of data regarding callus induction (%) reveals that the highest callusing was recorded in media supplemented with higher auxin: low cytokinin ratio. (4.0: 0.5 NAA: BAP ratio) (Plate 6c). Callusing involves a de-differentiation process where in pre-determined cells from different tissues are induced to multiply uncontrollably. A high auxin / cytokinin ratio is known to induce this phenomenon under right conditions in species belonging to diverse genera and families and similar trend was observed in the current study. Several workers have reported callus development in *Alstroemeria* from inflorescence bud explants or structures originating in floral tissue like mature embryos etc. Callus was obtained from mature embryos of *Alstroemeria cv*. Butterfly on MS media supplemented with 4 mg l^{-1} 2, 4-D combined with BAP 0-4 mg l^{-1} (Gonzalez 1990). With high auxin:cytokinin ratio, callus induction from floral explants in other monocots like Iris on MS medium + 2.0 mg l^{-1} TDZ and 3.0 mg l^{-1} NAA has been reported (Violeta *et al*., 2013). Hutchinson (1994) also recorded callus induction from mature zygotic embryos of a tetraploid Alstroemeria (*A.* pelegrina × *A.* psittacina). Gonzalez 1995 recorded callus induction on 40, 34, and 32 per cent of embryos cultured on MS medium supplemented with 4 mg 2, 4- D l^{-1}, 2 mg picloram l^{-1}and 4 mg picloram l^{-1}respectively.

Highest callus fresh weight explant $^{-1}$ was recorded on the same plant growth regulator combination 4.5: 0.5 NAA: BAP ratio. Auxins (besides helping in cell division) are known to increase cell volume by a process called acid growth where in cell wall loosens up through acidification thus allowing increase in volume through uptake of more water. The increased cell volume means increase

in individual cell weight and hence increased weight of the callus. Gonzalez (1995) reported increase in callus fresh weight on MS media supplemented with 2 mg picloram l^{-1}.

5.3 Experiment–III : Standardization of media and growth regulator combinations for culture establishment and proliferation in *Alstroemeria*

5.3.1 Culture establishment and proliferation

In the current study, culture establishment of *Alstroemeria cv.* Pluto was gauged in terms of per cent establishment, days to sprouting and number of sprouted buds in rhizome tips and rhizome section explants. Thirty-four (34) plant growth regulator combinations were tried and about 25 plant growth regulator combinations proved successful in culture establishment while as the remaining 9 plant growth regulator combinations failed to evoke any response (Table 4.2.1, 4.2.2 and Appendix-I). Rhizome tips and rhizome sections were incubated on MS-solid media, MS- solid media fortified with activated charcoal and MS- liquid media. MS- liquid media using filter paper bridges or sterilized hospital gauze fortified with BAP: IBA at 1.5: 0.2 mg l^{-1} proved best for culture establishment in terms of per cent establishment, days to sprouting and number of sprouted buds both in rhizome tips and rhizome sections (Tables 4.2.1 and 4.2.2). Culture establishment in rhizome tips was relatively better as compared to rhizome sections. This may be due to presence of an intact growing tip that is a source of auxin and hence a strong sink/growing area with better chances of continued growth and sprouting. Effect of Plant growth regulator combination on culture establishment in *Alstroemeria* have been reported by several workers. Klerk (2010) reported good establishment and subsequent growth in liquid media where explants are placed on Filter Paper Bridge. In this study establishment of explants on liquid media using sterile hospital gauze gave highest success in terms of per cent establishment, sprouting and number of sprouted buds. Lowest number of days to sprouting of rhizome tips and rhizome sections (9.18 and 9.98 days respectively and highest number of sprouted buds (4.05 and 3.00 in rhizome tips

and rhizome sections respectively) was recorded in liquid media. In this study IBA 0.2 mg l^{-1} was the most appropriate for increasing the number of sprouted buds in rhizome which may be due to reduced apical dominance.

In the current study dark coloured media fortified with 1000 mg l^{-1} activated charcoal was used to simulate photophobic conditions suitable for *Alstroemeria* rhizome growth in soil. Use of activated charcoal (1000 mg l^{-1}) gave comparable results with that of the liquid media. These are in terms of culture establishment and proliferation of *Alstroemeria*. No reports are available on use of activated charcoal darkened media for culture establishment and proliferation of *Alstroemeria* rhizomes. Further research is needed to explore the usefulness of activated charcoal in improving *in vitro* propagation in *Alstroemeria* particularly from rhizome tips and rhizome sections. In conformity to our findings Pierik *et al.* (1988) reported that rhizome multiplication required a cytokinin in the medium with BA being most effective.

Data on proliferation (Table 4.4.1) points to the usefulness of activated charcoal in media towards improving per cent proliferation, number of erect shoots, number of new rhizome buds, rhizome fresh weight and multiplication index. Data also reveals that increasing IBA concentration from 0.2-0.4 mg significantly improves number of erect shoots, number of new rhizome buds, rhizome fresh weight and multiplication index. Increased proliferation activity under higher IBA concentration may be due to reduced apical dominance. This is in contrast to reports by Pierik *et al*. (1988) who observed no significant effect of auxin on multiplication. However Pumisutapon *et al.* (2009) showed the role of IBA in enforcing apical dominance in his studies on decapitated shoots and rhizome sections. However, Gabryszewska (1985) recommended use of BA for tissue multiplication in *Alstroemeria.* Podwyszynska *et al*. (1997) recorded highest number of shoots with BA 6 mg l^{-1}.

5.4 Experiment – IV : Standardisation of rooting in *Alstroemeria*

5.4.1 Rooting

Influence of varying concentrations of IBA and NAA with or without Activated charcoal on rooting behaviour of *Alstroemeria cv*. Pluto in the current investigation was studied in terms of per cent rooting, days to appearance of root, number of roots and root length. Highest per cent rooting, number of roots and root length was recorded on sterile hospital gauze in MS- liquid medium supplemented with NAA 1.5 mg l^{-1}. In the current study liquid MS media supplemented with NAA 1.5 mg l^{-1} resulted in best rooting response in terms of per cent rooting (54.81), days to appearance of root (10.87), number of roots explant^{-1} (3.12) and root length (16.42 mm). Hakkart and Versluijs 1988 also recorded root formation was better on filter paper bridges in a liquid medium than on a solid medium. Pierik *et al*. (1988) reported best rooting of *Alstroemeria cv*. Toledo under the plant growth regulator combination NAA 0.5 mg l^{-1}. Pedraza *et al*. (2006) reported rooting of shoots was induced on MS liquid medium, either with or without plant hormones. Gabryszewska (1995) reported rooting was strongly influenced by NAA. Podwyszynska (1998) reported application of BA at low concentration with Paclobutrazole (0.1- 0.5 mg l^{-1}) in the presence of 1.0 mg l^{-1} NAA resulted in higher rooting ability of rhizomes. Growth retardants applied with NAA strongly stimulated root formation but suppressed their elongation. Kristiansen and Brandt (1999) recorded thick roots were only produced in the presence of NAA and not affected by sucrose treatment. Gabryszewska (1985) recorded that 1.0 to 16.0 mg l^{-1} NAA was recommended for rooting. In contrast to the current study were rooting with IBA was poor, Han *et al*. (1994), reported the most effective (for per cent rooting, number of roots and root length) was IBA at 3.0 mg l^{-1} in *Alstroemeria* cv's Othello, lilac, glory, Cyprus and Yellow prince.

Chapter – 6

SUMMARY AND CONCLUSION

The findings in the present investigation regarding *in vitro* propagation in *Alstroemeria cv.* Pluto is summarised in the following sections:

6.1 Experiment–I : Standardisation of disinfection protocol for various explants in *Alstroemeria cv.* Pluto

6.1.1.1 Surface sterilization, culture asepsis and survival

- Surface sterilization with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1 %) dip for 10 minutes and ethyl alcohol 70% for 1 minute gave highest per cent uncontaminated cultures 79.20, 68.08 per cent and 63.46, 61.63 per cent in case of rhizome tips and rhizome sections at 2 weeks and 4 weeks of culture respectively, which is statistically at par with 5 minute and 30 second dip in mercuric chloride 0.1 % and ethyl alcohol 70 %.
- Surface sterilization with Carbendazim 200 ppm for 15 minutes followed by mercuric chloride (0.1%) dip for 3 minutes gave highest per cent uncontaminated cultures 63.25, 60.15 per cent and 71.95, 63.40 per cent in case of shoot tips and shoot nodal segments at 2 weeks and 4 weeks of culture respectively, which is statistically at par with 5 minute dip in mercuric chloride 0.1%.
- Among the four explants that were tried, rhizome tips proved to be better for starting *in vitro* propagation. 68.08 per cent rhizome tips survived under Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1 %) dip for 10 minutes and ethyl alcohol 70 % for 1 minute.

6.2 Experiment–II : Standardization of media and growth regulator combination for culture establishment in *Alstroemeria cv.* Pluto

6.2.1 Culture establishment per cent in *Alstroemeria cv.* Pluto

- Rhizome tips proved better explants for culture establishment as compared to other explants tried.
- MS- liquid media proved best for culture establishment in terms of per cent establishment, days to sprouting and number of sprouted buds.
- MS-liquid media supplemented with BAP + IBA: 1.5 + 0.2 mg l^{-1} had maximum per cent establishment (89.42 and 56.13) along with lowest days to sprouting (9.18 and 9.98) and highest number of sprouted buds (4.05 and 3.00) in case of rhizome tips and rhizome sections respectively.
- MS-solid media supplemented with BAP + IBA + Activated charcoal: 1.0 + 0.2 +1000 mg l^{-1} had maximum per cent establishment (78.25 and 40.24 %) in case of rhizome tips and rhizome sections respectively.

6.3 Experiment-III: Standardisation of shoot proliferation in *Alstroemeria cv.* Pluto

6.3.1 Rhizome proliferation

- Rhizome tips proved better explants for proliferation.
- MS-solid media fortified with activated charcoal proved best for rhizome proliferation.
- MS-solid media supplemented with BAP + IBA + GA_3 + activated charcoal : 2.0 + 0.4 + 0.5 + 1000 mg l^{-1} revealed highest proliferation in terms of per cent proliferation (88.85), number of erect shoots (5.75), number of new rhizome buds (3.75), rhizome fresh weight/shoot complex (6.05g) and multiplication index/cycle (x2.76)

6.4 Experiment–IV: Standardisation of callus induction and organogenesis in *Alstroemeria cv.* Pluto

6.4.1 Callusing

- Callus induction was possible only in case of inflorescence bud explants.
- Inflorescence bud explants in MS-medium fortified with BAP + NAA: 0.5 + 4.0 mg l^{-1} revealed highest per cent callus induction (79.37).
- Highest mean callus fresh weight (2.98 mg) under NAA + BAP: 4 + 0.5 mg l^{-1} was significantly superior to all other NAA based growth regulator combinations.
- Friable white green callus was obtained in calluses growing in media supplemented with NAA.
- Attempts at regeneration of callus under various plant growth regulator combinations were unsuccessful

6.5 Experiment-V: Standardisation of rooting in *Alstroemeria*

6.5.1 Rooting in rhizome explants in *Alstroemeria cv.* Pluto

- Auxins (NAA) produced maximum results with respect to per cent rooting, number of roots and root length.
- MS-liquid media proved better in terms of rooting.
- Explants incubated in MS-liquid media supplemented with NAA 1.5 mg l^{-1} resulted in significantly higher per cent rooting (54.81).

CONCLUSION

i. Rhizome tips taken during the vegetative growth are suitable explant for starting the culture in *Alstroemeria cv.* Pluto.

ii. Maximum uncontaminated growing cultures were obtained with Carbendazim 200 ppm for 30 minutes followed by mercuric

chloride (0.1%) treatment for 10 minutes and final treatment with ethyl alcohol (70%) dip for 1 minute.

iii. Maximum culture establishment was obtained in MS- liquid media fortified with plant growth regulator combination BAP + IBA: 1.5 + 0.2 mg l^{-1}.

iv. Proliferation of rhizome tip explants in media fortified with activated charcoal was significantly higher as against the other treatments. [BAP + IBA + GA3 + Activated charcoal: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}].

v. Highest callus induction per cent was obtained with plant growth regulator combination BAP + NAA: 0.5 + 4.5 mg l^{-1}.

vi. Best rooting was recorded in MS- liquid media fortified with NAA 1.5 mg l^{-1}.

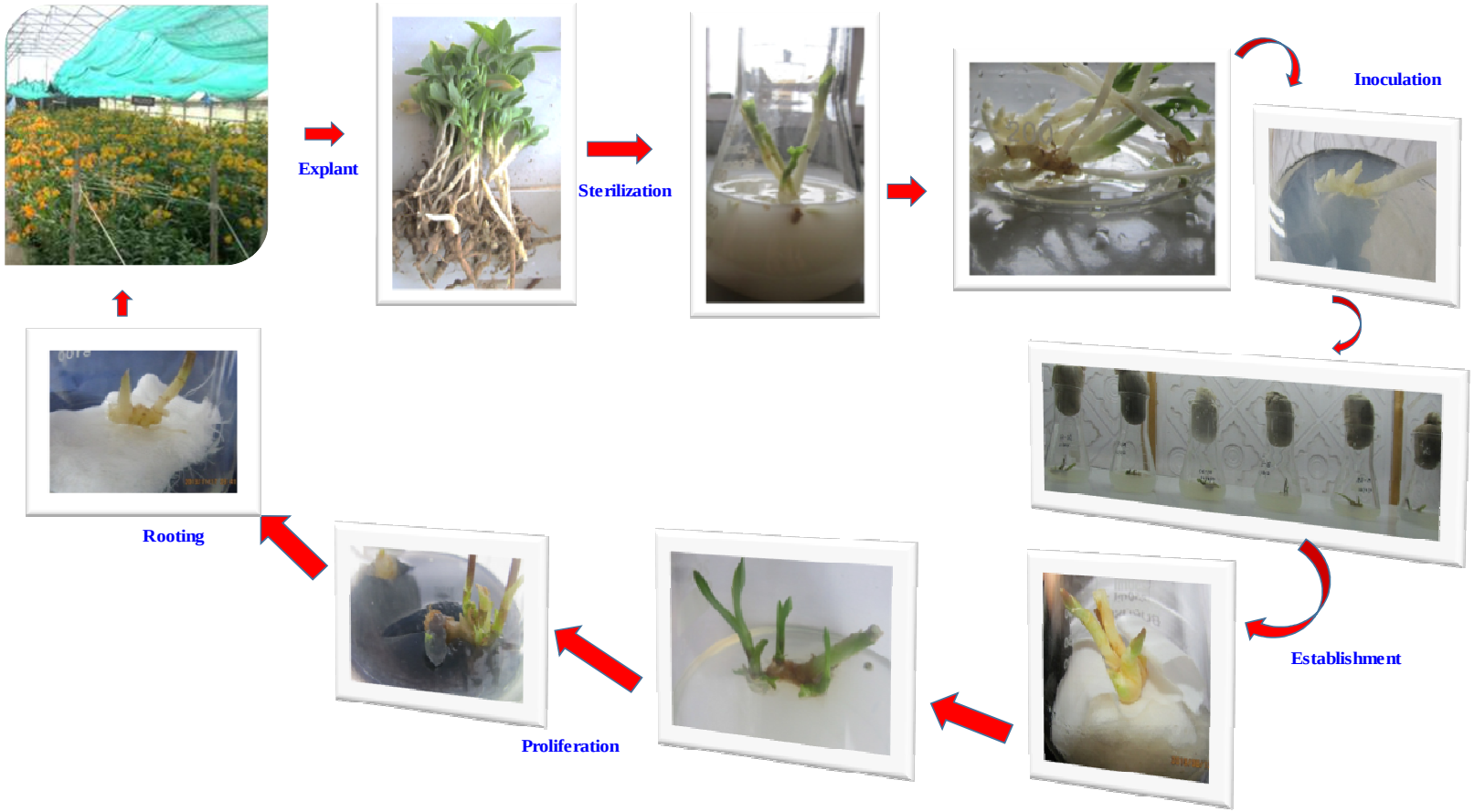


Plate-7 : Flow chart representing *in vitro* propagation protocol in *Alstroemeria cv.* Pluto

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Appendix – I

Growth regulator combinations with no response on culture establishment in *Alstroemeria cv.* Pluto

	MS + PGRs mg l^{-1}				
Treatment	**BAP**	**IBA**	**NAA**	**Kinetin**	**Activated charcoal**
T_1	0.2	0.02	-	-	-
T_2	1.5	0.2	-	-	-
T_3	2.5	0.4	-	-	-
T_4	2.5	0.5	-	-	-
T_5	3	0.5	-	-	-
T_6	0.5	-	-	-	-
T_7	1	-	-	-	-
T_8	1.5	-	-	-	-
T_9	-	0.2	-	1	-
T_{10}	-	-	0.2	0.5	-
T_{11}	-	-	0.2	1.5	-
T_{12}	1.5	-	0.4	-	1000

Appendix – II

Growth regulator combinations with no response on callusing in inflorescence bud explant of *Alstroemeria cv.* Pluto

Treatment	MS + PGRs mg l^{-1}			
	BAP	**2,4-D**	**NAA**	**GA_3**
T_1	0.2	2	-	-
T_2	0.3	2.5	-	-
T_3	0.4	3	-	-
T_4	0.5	3.5	-	-
T_5	0.5	4	-	-
T_6	0.8	6	-	-
T_7	1	6.5	-	-
T_8	0.3	-	2.5	-
T_9	0.5	-	5	-
T_{10}	-	1.5	1.5	-
T_{11}	-	-	4	-
T_{12}	0.5	-	0.4	0.5

Appendix – III

Plant growth regulators tried for callusing in leaves and shoot nodal segments of *Alstroemeria Cv.* Pluto showing no response

Treatment	MS + PGRs mg l^{-1}			
	BAP	**2,4-D**	**NAA**	**GA_3**
T_1	0.2	2	-	-
T_2	0.3	2.5	-	-
T_3	0.4	3	-	-
T_4	0.5	3.5	-	-
T_5	0.5	4	-	-
T_6	0.8	6	-	-
T_7	1	6.5	-	-
T_8	0.2	-	2	-
T_9	0.3	-	2.5	-
T_{10}	0.4	-	3.5	-
T_{11}	0.5	-	4	-
T_{12}	0.5	-	4.5	-
T_{13}	0.5	-	5	-
T_{14}	-	1.5	1.5	-
T_{15}	-	-	4	-
T_{16}	0.5	-	0.4	0.5

Appendix – IV

Plant growth regulator combinations tried for regeneration in inflorescence bud callus of *Alstroemeria cv.* Pluto showing no response

Treatment	MS + PGRs mg l^{-1}		
	BAP	**2,4-D**	**NAA**
T_1	1.0	0.5	-
T_2	2.0	0.5	-
T_3	3.0	0.5	-
T_4	5.0	0.5	-
T_5	1.0	-	0.5
T_6	2.0	-	0.5
T_7	3.0	-	0.5
T_8	5.0	-	0.5

Appendix – V

Growth regulator combinations tried with no response in terms of proliferation

Treatment	MS + PGRs mg l^{-1}					
	IBA	**GA_3**	**Kinetin**	**TDZ**	**NAA**	**Activated charcoal**
T_1	0.2	0.5	1.0	-	-	-
T_2	0.2	-	1.5	-	-	1000
T_3	0.2	-	2.5	-	-	-
T_4	-	0.5	1.5	-	0.2	1000
T_5	-	-	2.5	-	0.2	-
T_6	-	-	3.0	-	0.2	-
T_7	0.2	-	-	0.02	-	-
T_8	0.2	0.5	-	0.05	-	1000
T_9	-	-	-	0.02	0.2	-
T_{10}	-	0.5	-	0.05	0.2	1000

Appendix – VI

Treatments with no response on rooting in rhizome explants in *Alstroemeria cv.* Pluto

Treatment	MS + PGRs mg l^{-1}		
	IBA	**NAA**	**Activated charcoal**
T_1	0.5	-	1000
T_2	-	0.5	-
T_3 (Liquid)	1	-	1000

ANOVA

Exp. 1: Influence of sterilant treatments on culture asepsis (%) of rhizome tip explant (at 2 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	7	2387.0	20.71	15.66
Error	24	115.2		
Total	31			

Influence of sterilant treatments on culture asepsis (%) of rhizome tip explant (at 4 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	7	1853.3	24.77	12.62
Error	24	74.8		
Total	31			

Influence of sterilant treatments on culture asepsis (%) of rhizome section explants (at 2 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	7	1665.7	12.27	17.00
Error	24	135.8		
Total	31			

Influence of sterilant treatments on culture asepsis (%) of rhizome section explant (at 4 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	7	1612.5	15.54	14.86
Error	24	103.8		
Total	31			

Influence of sterilant treatments on culture asepsis (%) of shoot tip explants (at 2 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	5	119.52	4.32	7.81
Error	18	27.65		
Total	23			

Influence of sterilant treatments on culture asepsis (%) of shoot tip explants (at 4 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	5	104.04	3.86	7.71
Error	18	26.95		
Total	23			

Influence of sterilant treatments on culture asepsis (%) of shoot nodal segments (at 2 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	5	194.42	3.35	11.32
Error	18	58.09		
Total	23			

Influence of sterilant treatments on culture asepsis (%) of shoot nodal segments (at 4 weeks of culture) of *Alstroemeria cv*. Pluto

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	5	242.81	7.50	8.45
Error	18	32.39		
Total	23			

Influence of sterilant treatments on survival (%) of rhizome tip explants of *Alstroemeria cv.* Pluto

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	7	1562.5	16.24	14.31
Error	24	96.2		
Total	31			

Influence of sterilant treatments on survival (%) of rhizome section explants of *Alstroemeria cv.* Pluto

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	7	1765.4	16.98	14.87
Error	24	103.9		
Total	31			

Influence of sterilant treatments on survival (%) of shoot tip explants of *Alstroemeria cv.* Pluto

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	5	298.85	11.22	7.66
Error	18	26.64		
Total	23			

Influence of sterilant treatments on survival (%) of shoot nodal segment explants of *Alstroemeria cv.* Pluto

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	5	140.38	4.63	8.17
Error	18	30.30		
Total	23			

Exp. II: Influence of growth regulator combinations on culture establishment of rhizome tips in *Alstroemeria cv.* Pluto

Percent establishment

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	24	1311.5	63.85	6.37
Error	75	20.5		
Total	99			

Influence of growth regulator combination on days to sprouting and number of sprouted buds in *Alstroemeria cv.* Pluto

Days to rhizome sprouting in rhizome tips

Analysis of Variance for days to sprouting in rhizome tips

Source	DF	MS	F	C.D
Treatment	21	25.429	125.30	0.63
Error	66	0.203		
Total	87			

Sprouted buds in rhizome tips

Analysis of Variance for sprouted buds in rhizome tips

Source	DF	MS	F	C.D
Treatment	21	1.5675	9.81	0.54
Error	66	0.1598		
Total	87			

Influence of growth regulator combinations on culture establishment of rhizome sections in *Alstroemeria c*

Percent establishment

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	24	251.47	12.83	6.23
Error	75	19.68		
Total	99			

Influence of growth regulator combination on days to sprouting and number of sprouted buds in rhizome sections in *Alstroemeria cv.* Pluto

Analysis of Variance for days to rhizome sprouting

Source	DF	MS	F	C.D
Treatment	21	22.395	10.82	2.03
Error	66	2.070		
Total	87			

Analysis of Variance for number of sprouted buds

Source	DF	MS	F	C.D
Treatment	21	1.5675	9.81	0.56
Error	66	0.1598		
Total	87			

Exp 3: Influence of growth regulator combination on callusing in inflorescence bud explant of *Alstroemeria cv.* Pluto

Callus induction percent

Analysis of Variance for transformation

Source	DF	MS	F	C.D
Treatment	3	2610.6	17.04	19.06
Error	12	153.2		
Total	15			

Callus fresh wt.

Analysis of Variance for callus fresh weight (mg).

Source	DF	MS	F	C.D
Treatment	3	3.7714	34.50	0.50
Error	12	0.1093		
Total	15			

Exp 4: Influence of growth regulator combinations on proliferation in rhizome tip explants of *a cv.* Pluto

Percent proliferation

Analysis of Variance for Transformation

Source	DF	MS	F	C.D
Treatment	6	2127.9	15.49	17.23
Error	21	137.3		
Total	27			

Number of erect shoots

Analysis of Variance for number of erect shoots

Source	DF	MS	F	C.D
Treatment	6	8.405	7.51	1.55
Error	21	1.119		
Total	27			

Number of new rhizome buds

Analysis of Variance for new rhizome buds

Source	DF	MS	F	C.D
Treatment	6	5.3006	6.70	1.30
Error	21	0.7917		
Total	27			

Rhizome fresh weight / shoot complex

Analysis of Variance for fresh weight/shoot complex

Source	DF	MS	F	C.D
Treatment	6	3.2040	48.25	0.37
Error	21	0.0664		
Total	27			

Multiplication index

Analysis of Variance for multiplication index

Source	DF	MS	F	C.D
Treatment	6	2.9514	33.38	0.43
Error	21	0.0884		
Total	27			

Exp. 5 : Influence of growth regulator combinations on rooting in rhizome explant in *Alstroemeria* cv. Pluto

Percent rooting

Analysis of Variance for Trar

Source	DF	MS	F	C.D
Treatment	9	341.97	6.48	10.49
Error	30	52.78		
Total	39			

Days to appearance of root

Analysis of Variance for days to appearance of root

Source	DF	MS	F	C.D
Treatment	9	6.52	0.22	NS
Error	30	29.96		
Total	39			

Number of roots

Analysis of Variance for no. of roots

Source	DF	MS	F	C.D
Treatment	9	1.7466	2.04	1.33
Error	30	0.8545		
Total	39			

Root length

Analysis of Variance for root length

Source	DF	MS	F	C.D
Treatment	9	8.332	1.03	NS
Error	30	8.065		
Total	39			

Sher-e-Kashmir
University of Agricultural ε Technology of Kashmir
Division of Floricultur al & Aromatic Plants
Shalimar Campus, Srinagar – 190 025
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CERTIFICATE

Certified that all the corrections/amendments as suggested by

External Examiner Dr. Y.C. Gupta, Prof. & Head, Department of Floriculture and Landscaping, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P) during Viva-Voce examination held on August 8, 2014 have been incorporated in the manuscript entitled **"Studies on *in vitro* propagation in *Alstroemeria* cv. Pluto"** submitted by **Ms. Ambreena Din (Regd. No. 2012-A-918-M).**

(Dr. Imtiyaz T. Nazki)
Chairman
Advisory Committee