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Developmental anatomy and cryopreservation of structures created during indirect organogenesis in a Eucalyptus grandis / Eucalyptus urophylla hybrid

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Developmental

Anatomy and Cryopreservation

of

Structures created during

Indirect Organogenesis

in a

Eucalyptus grandis/ Eucalyptus urophylla hybrid

By

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Submitted in partial fulfilment of the requirement for the degree of Masters of Science in the School of Animal, Plant and Environmental Sciences.

University of Witwatersrand, Johannesburg. 2009

Declaration

I declare that this dissertation is my own work. It is being submitted for the Degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any University.

Signature of candidate

Date

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ABSTRACT

Callus was initiated from *Eucalyptus grandis x urophylla* axillary bud segments and cultured on a callus induction medium to regenerate shoots. At the gross morphological level two types of callus were described; a crystalline callus and compact glassy callus (yellow, white and brown) that gradually became nodular. A red callus was also identified in the late stages of development (between days 43 and 57). The crystalline and compacted callus sometimes formed dark spots on the surface which were possibly an indication of the accumulation of tannins. Histologically callus developed two types of cells; small compact morphogenic cells and loosely arranged large nonmorphogenic cells. The morphogenic cells were meristematic in appearance and presumed to be involved in the formation of shoot-bud structures. The meristematic activity produced vascular nodules (nodular structures) which differentiated into shoot-buds. The vascular tissue of callus was continuous with the developed shoot-buds. This showed that the shoots were initiated from the differentiation of callus and not from the original axillary bud merisitems. The process of shoot-bud development was monitored for 8 weeks with 39% of the material producing shoots. The induced shoots were then cultured on a root induction medium. Results indicated that root initiation occurred within 24 hours of culture. Roots were either formed from callus re-differentiation at the base of the shoot or from the shoot vascular cambium. Vascular connections that developed in the adventitious roots formed from parenchyma differentiation. Another connection was formed between the lateral root primordium and primary root. The total yield of shoots that formed roots was 74% over a four-week period. Although cells that formed the adventitious roots were identified, the initial stages of root initiation were not recorded. Callus that was 22 days old was cryostored using both slow and rapid freezing rates and the subsequent survival determined by tetrazolium chloride testing (viability) and the potential to regenerate shoots (post-thaw growth). The callus (slow freezing rate) that was pretreated by exposure to cryoprotectants and then dried for 20 minutes showed 55% viability compared with callus that was dried first and then exposed to cryoprotectants which had a 52% viability during slow freezing. The material that was rapidly frozen

when was not viable. None of the cryo-treatments resulted in postthaw growth. The results indicate that the slow freezing method has the potential to be the method for cryostorage of callus of *Eucalyptus*.

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LIST OF ABBREVIATIONS

^{o}C	degrees celsius
^{o}C/sec	degrees per seconds
F	fahrenheit
g/ l	grams per litre
ml/l	millilitres per litre
Fe	iron
Cu	copper
Zn	zinc
B	boron
Mo	molybedium
N	nitrogen
P	phosphorus
K	potassium
$HgCl_2$	mercuric chloride
$Ca(OCl)_2$	calcium hypochlorite
v/v	volume by volume
%	percent
Tween 20	polyoxyethylene sorbitan monolaurate
ml	milliliters
KH_2PO_4	potassium dihydrogen orthophosphate
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
NAA	α-napthaleneacetic acid
BAP	6-benzylaminopurine
μgml^{-1}	microgram per milliliter
KPA	kilopascal
pH	hydrogen ion concentration
$\mu gmol\ m^{-2}\ s^{-1}$	micromoles per metre squared per second
PPFD	photosynthetic photon flux density
GU	*Eucalyptus grandis x urophylla*
MS	Murashige ans Skoog (1962) basal nutrients
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid

cm	centimeter
mg/l	milligrams per litre
mm	milllimetres
h	hours
CO_2	carbon dioxide
C m^3	Centimetre cubed
m^3	metres cubed
DPX	di-n-butylphthalate in xylene
VCD	Vinylcyclohexene
NSA	hardener:nonelyl succinic anhydride
DMAE	accelerator:dimethylaminoethanol
DMSO	dimethysulfoxide
MDA	malondialdehyde
MEL	meristem electrolyte leakage
EG	ethylene glycol
PEG	polyethylene glycol

1. INTRODUCTION

1.1 Forestry in South Africa

The South African forests are mainly composed of savannas, indigenous forests and plantations (Shackleton *et al*., 2007). Grasses and trees which are the main constituents of savannas are estimated to cover 42 million hectares of South Africa (Thompson *et al*., 2001), whilst indigenous forests area is approximately 350 000 hectares (Shackleton *et al*., 2007). Plantation forestry (1.3 million hectares) forms an essential part in the South African Forestry industry and of the economy of the country (Anonymous, 2002; Anonymous, 2005). This is particularly due to the considerable growth of the pulp and paper sectors of the industry (Pallet and Sale, 2004).

The South African plantations are comprised of both soft woods (e.g. *Pinus* species) and hardwoods such as *Eucalyptus* and *Acacia* species (Cellier, 1999; Meadows, 1999; Chamberlain *et al*., 2005b; Tewari, 2005). The *Pinus* and *Eucalyptus* plantations are dispersed along the eastern and south-eastern parts of the country (Van Staden *et al*., 2004). *Eucalyptus* species are mainly planted in low areas of KwaZulu Natal and Mpumalanga (Anonymous, 2004). Pine trees are mostly distributed in the southern Cape, but can also be grown in Mpumalanga and the cooler areas of KwaZulu Natal (Anonymous, 2004). The softwoods are harvested for pulp between 12 and 15 years or for sawlogging which usually is between 27 and 30 years (Chamberlain *et al*., 2005b). The hardwoods e.g. *Eucalyptus* trees are ready to be harvested between 7-10 years for pulp (Chamberlain *et al*., 2005a).

Eucalyptus species are amongst the most important plantation hardwoods in the world (Turnbull, 1991; Myburg *et al*., 2006). The genus belongs to the family Myrtaceae, which comprises more than 700 species (Brooker, 2000; Ladiges *et al*., 2003). Members of *Eucalyptus* are frequently grown in the tropical and sub-tropical countries although they are endemic to Australia and the islands to its North including Timor, New Guinea and the Phillipines (Cremer, 1969; Eldridge *et al*., 1993; Myburg *et al*., 2006). *Eucalyptus* is used

for timber, pulp and paper production, charcoal, mine props, poles and firewood. Members of the genus are also used commercially for the production of essential oils which are extracted from the leaves (Durand-Cresswell *et al*., 1982; Turnbull, 1991; Bandyopadhyay *et al*., 1999; Myburg *et al*., 2006).

1.2 Propagation practices

Eucalyptus trees are propagated by two methods: sexual propagation or via vegetative (asexual/cloning) propagation.

1.2.1 Sexual Reproduction

Propagation by seeds

In sexual propagation, plants are formed when two gametes unite to form a zygote that develops into an embryo (i.e. in seeds), which will eventually develop into a plant (George, 1993). In forestry, sexual propagation has disadvantages, *viz.* it is a slow process because it has a long juvenile phase and heterozygosity (undesirable in clonal propagation) might occur in seeds (Tzfira *et al*., 1998; Vengadesan *et al.*, 2002). Furthermore, forest tree traits have to be assessed at maturity and the long juvenile phase delays the process in traditional breeding and the size of forest trees and large areas used to conduct field trials create difficulties in assessing their performance (Tzfira, *et al*., 1998).

Sexual Hybridisation

Hybridisation entails breeding of elite trees through careful combination of desired traits and genotypes of individual species (De Assis, 2000). By the use of controlled pollination, hybrids are produced as seedlings, which undergo genetic field tests (Tzfira., *et al*., 1998). The superior hybrids are further multiplied through vegetative propagation methods such as cuttings or *in vitro* approaches (Tzfira., *et al*., 1998). Breeding and domestication of

Eucalyptus commenced in South Africa in 1984 (Denison and Kietza, 1993a, 1993b). Subsequently conventional tree breeding has formed a major part of the forestry industry in order to improve the characteristics of the wood produced from plantations, to improve seed quality (Raymond and Apiolaza, 2004), and to produce genotypes suitable to different environmental conditions and marginal lands.

Hybrid vigour and crossing of traits of *Eucalyptus* species is usually achieved through interspecific hybridisation (Verryn, 2000). In South Africa, such breeding programs (i.e. hybridization) have been used commercially to improve different properties of *Eucalyptus* species, such as wood and pulp properties, drought and cold tolerance, rapid growth, disease resistance and high rooting capabilities (Verryn, 2000; 2002). *Eucalyptus* species with desirable characters (Table 1) have been crossed to form hybrids that can be cloned for further mass production (Verryn, 2000). In addition, *in vitro* propagation methods have been applied in the breeding programs to rapidly capture the total genetic gain and also allow uniformity (Campbell *et al*., 2003; El-Kassaby and Moss, 2004). The genetically improved plants produced *in vitro* are used as starting material for micropropagation using organogenesis and somatic embryogenesis (Park, 2002). Such superior genotypes can be conserved or field-tested for commercial suitability (Park, 2002).

Eucalyptus grandis (Hill ex Maiden) has been the most planted hardwood in the South African forestry sector, covering over an area of 311 000 hectares (Pierce and Verryn, 2001; Anonymous, 2003). It is extensively planted for pulpwood and mining timber (Anonymous, 2002) and because of its good quality of wood and rapid growth (Eldridge *et al*., 1993). Due to its superior growth characteristics *Eucalyptus grandis* can be hybridised with other *Eucalyptus* species, for instance, *E. camaldulensis, E. nitens, E urophylla* (Anonymous, 1999). *Eucalyptus urophylla* is native to Indonesia and has important properties such as disease resistance (Eldridge *et al*., 1994) and rapid growth (Wright, 1997). *E. urophylla* has been widely established in South Africa, Brazil, China, Congo and some other countries (House and Bell, 1994; Wright, 1997).

Table 1: Characteristics of various *Eucalyptus* species favourable to forestry (Verryn, *et al*., 1999)

Species	Vigour (heterosis)	Drought tolerance	Cold tolerance	Disease resistance	Rooting capability	Termite resistance
E. grandis	High	Low	Low	Low	Fair	High
E. urophylla	Fair	Fair	Low	High	Fair	Fair
E. nitens	High	Low	High	Fair	Low	Low
E. camaldulensis	Low	High	Fair	Fair	High	Low

In countries such as Brazil, hybridisation has been successful (e.g. *Eucalyptus grandis x Eucalyptus urophylla*) in improving cellulose and paper production (Bison *et al*., 2007). The hybridisation between *E. grandis* and *E. urophylla* has been shown to produce trees that are resistant to canker disease and to produce higher quality wood (Brandão *et al*., 1984).

1.2.2 Vegetative propagation

Vegetative propagation of plants *in vitro* is practised in agriculture, horticulture and forestry (Vengadesan *et al*., 2002). Vegetative propagation is important in forestry as it allows for the propagation of improved superior trees. This practice also offers uniformity in the regenerated plantlets and mass propagation of similar genotypes (Merkle and Dean, 2000; Park, 2002). The most common method of vegetative propagation of *Eucalyptus* is via cuttings (Titon *et al*., 2006). However cuttings of non-juvenile shoots in woody/forest trees are prone to recalcitrance to regenerate roots (Greenwood *et al*., 2001).

1.3 The Process and Application of *in vitro* propagation

The applications of the *in vitro* propagation of woody plants such as *Eucalyptus* can be achieved through the application of mass production of selected genotypes, conservation of the germplasm and the genetic engineering of the germplasm (Jiménez, 2001; Ramage and Williams, 2002).

1.3.1 Mass production of selected genotypes

In vitro culture of plantation germplam is utilised to mass-propagate improved or selected genotypes and provide genetically uniform clonal material. The material can be used for breeding purposes, seed orchards, plantations, production of transgenic trees and conservation (Pijut *et al*., 2007). For example, *in vitro* methods can be used with cutting propagation to improve the capability of difficult-to-root tree species (Yasodha *et al*., 1997). In the forestry industry, the most utilized routes of morphogenesis of *in vitro culture* are organogenesis and somatic embryogenesis.

The most common obstacles that are associated with *in vitro* culture of forest species is recalcitrance (e.g. inability to root) and somaclonal variation. Recalcitrance is here defined as failure of plant cells, tissues or organs to react to tissue culture manipulations (Benson, 2000). Recalcitrance is considered to occur due to the inappropriate selection of the explant tissues or the stock plant e.g. age of donor, morphology and growth stage, contaminants and toxic metabolites released during injury of tissues (Fay *et al.*, 1999). Somaclonal variation can be caused by two factors *viz*., epigenetic and genetic conditions (Jayasankar, 2000; Kaeppler *et al.*, 2000; Jain 2001). These conditions seem to be associated with the age of the plant, explant type, genotype, and the culture conditions (Veilleux and Johnson, 1998). Stimulation of epigenetic variation can be caused by conditions such as prolonged subculture cultures which lead to habituation of the cultures which similarly introduces variations (Jayasankar, 2000; Kaeppler *et al*., 2000). Changes that are associated with genetic variation are chromosome modification (addition and deletion); gene mutation, gene activation and gene

inactivation (Kaeppler *et al*., 1998; Jayasankar, 2000). The stimulation of somaclonal variation can be used as a technique to produce desired traits in recalcitrant forest tree germplasm in which variation cannot be induced through traditional breeding programmes (Huang *et al*., 1993). The disadvantages associated with this technique include low frequency of variants; some of the induced variants may not be true variants being epigenetic in nature and others may not be desirable (Huang *et al.*, 1993).

1.3.1.1 Somatic embryogenesis and Organogenesis

Somatic embryogenesis

Somatic embryogenesis is a developmental process which occurs when embryos arise from non-zygotic cells without vascular association with the parental tissue (Schaeffer, 1990; Emons, 1994; Raemakers *et al*., 1995; Von Arnold *et al*., 2002). Somatic embryos can develop directly from individual somatic cells, tissues from the explant or indirectly from dedifferentiated callus cells (Murashige, 1978; Williams and Maheswaran, 1986; Hansen and Wright, 1999).

Mass propagation via indirect embryogenesis has the advantage that a high frequency of regenerated plantlets can be achieved (Merkle and Trigiano, 1994; Jain, 1999; Merkle, 1999; Merkle and Dean, 2000). For instance bioreactors using liquid cultures can be used to proliferate large numbers of embryos (Merkle and Trigiano, 1994; Jain, 1999). Furthermore, as a result of embryo conversion, shoots and roots are formed simultaneously in a one-step process, and this is less time consuming for mass production than the organogenesis approach (Merkle and Trigiano, 1994; Jain 1999; Pinto *et al*., 2002, 2008). Although propagation via embryogenesis results in a high rate of production in many species, particularly ornamentals, there are exceptions (e.g. Pine), which exhibit a low frequency of plantlet production (Jain, 1999; Merkle, 1999; Merkle and Dean, 2000). Additionally a particular disadvantage of this method is the risk of somaclonal variation and this has hindered the

application and commercialisation of the approach to forest tress (Merkle, 1995, 1999; Jain, 2006).

Nevertheless, somatic embryogenesis has the potential to be used as a valuable technique for clonal multiplication of *Eucalyptus* species (e.g. Muralidharan *et al* 1989; Watt *et al*., 1991; Muralidharan and Mascarenhas, 1995; Bandyopadhyay *et al*., 1999). However it has not been commercially applied to the genus because of deficient knowledge of the somatic embryogenic system to date and because the yields are generally low (Watt *et al*., 1999; Pijut *et al*., 2007).

Organogenesis

Organogenesis is the formation and development of plant organs and this generally involves the formation of shoots, roots, tubers and flowers directly from pieces of tissue of the original parent plant or indirectly from callus (George, 1993; Schwarz and Beaty, 2000). Axillary bud multiplication, which occurs directly, is one method that is used for the propagation of *Eucalyptus* (Watt *et al.,* 1997; Watt *et al*., 2001). This method can guarantee that there is little possibility of somaclonal variation of the plantlets since shoots are formed from preformed bud meristems (Fay, 1992; Merkle and Trigiano, 1994; Rani and Raina, 2000).

Indirect organogenesis, morphology and morphogenesis

During indirect organogenesis, the nascent organs are adventitious in nature and generally originate from callus induced from shoots, roots and nonzygotic embryos (Christianson and Warnick, 1988; George, 1993; Schwarz and Beaty, 2000). Callus is an amorphous mass of loosely arranged parenchyma cells which can be formed *in vitro* from rapidly dividing cells of the parent tissue (Yeoman, 1970; Yeoman and Macleod, 1977; Caponetti, 2000). A callus can also be induced by wounding or by the invasion of the plant tissues by microorganisms (Braun, 1954; George, 1993). Callus formation is usually achieved *in vitro* by the use of plant growth regulators such as 2,4-D

(Ahloowalia *et al*., 2004), 1-naphthaleaneacetic acid (NAA) and indole-3-acetic acid (IAA), (George, 1993). Successful callus initiation is dependent on the source of explant, the type of medium and culture conditions (Constabel, 1984). The structure and colour of a callus varies depending on the plant species and source tissue. A callus can be soft or hard in texture and generally varies from green to yellow to white (Pierik, 1987). Subculturing of callus to fresh medium is important since maintaining the callus for a long time on the same medium may lead to: alteration in the morphology of the callus, reduction in the ability of the tissue to regenerate (Murashige, 1978; Thorpe, 1982), production of toxic metabolites, nutrient depletion and dehydration of the agar (Dodds and Roberts, 1985). As previously discussed, callus cultures are prone to somaclonal variation and this can cause variation in the produced plant organs (Schwarz and Beaty, 2000; Pijut *et al*., 2007).

1.3.1.2 Ontogeny of organogenesis

Plants organs can be formed via three different vascular differentiation processes, viz. primary, secondary (Ye, 2002; Aloni *et al*., 2006) and regenerative differentiation (Sachs, 1981; Aloni and Plotkin, 1985; Aloni, 2004). In primary differentiation vascular tissues and plant organs are formed from a particular procambium, while plant development in the secondary differentiation occurs from the vascular cambium (Ye, 2002; Aloni *et al*., 2006; Bao, 2008). In early stages of organ development xylem and phloem tissues are initiated by differentiation from procambium and vascular cambium which are essentially meristematic in nature (Foster, 1952; Ye, 2002; Carlsbecker and Helariutta, 2005). Meristems are cells that form part of plant growth by undergoing mitotic cycles and eventually differentiate into organs (Steeves and Sussex, 1989; Trigiano and Gray, 2005). Meristematic regions arise either by multiplication of existing meristems (e.g axillary meristem) or by adventitious meristem formation from other plant tissues or cells (e.g. callus cells) (Warren, 1991; Romberger *et al*., 1993). Apical meristem cells are produced at the plant apices and form part of the primary shoot and root meristem (Kerstetter and Hake, 1997).

On the other hand, during regenerative differentiation cells that are parenchymatous in nature become meristematic and it is from these cells that the vascular tissues develop (Aloni, 1980; Aloni and Plotkin, 1985). The vascular tissues then form a continuous system to distribute water, nutrients and photosynthetic material to all the intact parts of the plant (Esau, 1965; Aloni, 1987; Nelson and Dengler, 1997; Aloni, 1995; Mattsson *et al*.; 1999; Ye, 2002). The components of xylem are: conducting tracheary elements, xylem fibres and non-conducting parenchyma; whilst phloem is made-up of conducting sieve elements, non-conducting parenchyma cells and fibres (Carlsbecker and Helariutta, 2005). The xylem conducts water, nutrients and plant growth regulators to all parts of the plant and gives support to the plant. The phloem tissue is responsible for distribution of food material (Aloni, 1995; Berlerth *et al*., 2000; Turner and Sieburth, 2002; Ye, 2002).

Shoots and roots

Shoot buds arise from primary shoot apical meristems, axillary meristems and adventitious meristems (Randall *and* Kerstetter, 1997; Shimizu-Sato and Mori, 2001). Shoot apical meristem cells can be initiated during *in vitro* organogenesis to produce primary shoot buds and axillary meristems are formed in the axils of leaves and produce axillary shoot buds (Randall *and* Kerstetter., 1997; Shimizu-Sato and Mori, 2001). Shoots that are adventitious in nature, e.g. those initiated from organs such as stem or leaves may arise from epidermal or subepidermal cells (Romberger *et al*., 1993; Kantia and Kothari, 2002). In this regard, in a *Eucalyptus gunni* clone, shoot buds developed from a protuberance that formed from cultured leaves, which contained proliferating epidermal and subepidermal cells (Hervé *et al*., 2001). Parenchyma cells and tracheary elements were visible in the middle of the protuberance (Hervé *et al*., 2001). Similar studies done by Wagley *et al* (1987), on *Pinus eldarica*, indicated the shoot buds developed from the callus cells with traces of dividing cells on the surface of callus. The organization of both vascular and epidermal cells was also visible in all of the developmental stages of the shoot buds. In micropropagation, production of shoots via

indirect organogenesis can be achieved by increasing the content of cytokinin or decreasing auxin concentration in the culture medium (George, 1993).

Primary roots (e.g. those found in germinating seeds) arise from the meristems of the radicle or root apex of the embryo (Fahn, 1967; Clowes, 1969; Laskowski *et al*., 1995). Stangler in 1956 showed that the adventitious roots are initiated next to the vascular tissues (cambium). Adventitious roots in woody plants are usually derived from parenchymatous cells, phloem parenchyma, parenchymatous vascular rays and cambium of stems (Hartmann *et al*. 2002). Subsequent studies that have shown that adventitious root formation is associated with dedifferentiation of cells such as parenchyma tissues or shoot cambium that are usually situated around or next to the vascular tissue (Esau, 1965; Clowes, 1969; Soh and Bhojwani., 1999). Adventitious rooting has been exploited in the Forestry and Horticulture industries in breeding of trees and multiplying desired genotypes (Altamura, 1996). This method is used in areas of vegetative propagation namely *in vitro* rooting of adventitious clones and rooting of seedlings and cuttings (Fahn, 1967; Romberger *et al*., 1993; Altamura, 1996). Examples of studies that have been conducted with *Eucalyptus* species include: Sasse and Sands, 1997 (*E. globulus*), Hajari *et al*., 2006 (*E. grandis* clones).

1.3.2 Germplasm Conservation

Plant germplasm can be conserved using two broad approaches i.e. *in situ* and *ex situ* conservation. (Laliberté, 1997; Uyoh *et al*., 2003; Rao, 2004; Sarasan *et al*., 2006).

1.3.2.1 *In situ* conservation

In situ methods preserve genetic resources in their native habitats or protected regions (Johnson *et al*., 2001; Rao, 2004). There are a number of difficulties associated with the *in situ* conservation of plant germplasm. For instance, the germplasm may be threatened by environmental stresses. Likewise, the introduction of diseases, microbes, pests can negatively affect

the conservation. Furthermore this approach is labour intensive due to high spatial requirement and thus expensive to maintain (Engelmann, 2004).

13.2.2 *Ex situ* conservation

i. Botanic gardens- Plant material conserved in botanical gardens are available for educational, research and commercial purposes (Given, 1994; Hawkesworth, 1995; Lalibertè, 1997). This approach also offers protection of endangered and rare wild plants (Lalibertè, 1997). Botanical gardens also accommodate species with recalcitrant seed (Engels and Engelmann, 1999).

ii. Field genebanks-Field gene banks usually contain collections of recalcitrant seed producing species and vegetatively propagated species (e.g. *Eucalyptus* species) that can be available commercially for research or for distribution (Krishnapillay and Engelmann, 1996; Lalibertè, 1997; Uyoh *et al*., 2003). Disadvantages associated with field gene banks are that a large area of land is used and water restrictions and in some cases good maintenance and protection against natural disasters such as pests, diseases, natural hazards are required (Lalibertè, 1997, Uyoh *et al*., 2003). In South Africa policies have been imposed on the forestry industry in terms of saving land and water and this has impacted on the *in situ* conservation of potential valuable genotypes (Tewari, 2000).

iii. Seed stores- Seed banks are ideal for long-term storage of orthodox seeds under the specific conditions of low temperature (-20º C) and reduced moisture content (Ford-Lloyd and Jackson, 1990; Lalibertè, 1997). The seeds are collected, dried and stored (e.g. in liquid nitrogen at -196 º C) (Sakai, 2004). Regeneration and germination assessments must be carried out continuously during the storage period to test for vigour and retained viability. For seeds of *Eucalyptus,* although they are orthodox, and hence storable, this method is not ideal because they are prone to genetic variation when stored for protracted periods (Jarret and Florkowski, 1990, Pita *et al*., 1997). Recalcitrant seeds cannot be successfully stored in conventional seed banks

because the seeds cannot withstand the dry and cold conditions of conventional seed storage (Lalibertè, 1997).

iv. Gene banks-This method can be used to integrate genepools e.g. species with recalcitrant seeds and not suitable for seed banking and those that are from threatened populations (Shaanker *et al*., 2002).

1.3.2.3 *In vitro* storage

The *in vitro* storage method can be applied to all germplasm but is particularly useful for the conservation of plants that produce recalcitrant seeds, plants that are asexually propagated and the storage of spores, pollen, anthers, and protoplasts (Heywood and Iriondo, 2003; Rao, 2004). The ultimate aim of *in vitro* storage is to extend the germplasm life (Touchell and Dixon, 1999). Plant material stored by the *in vitro* method by necessity of the technique, must be free of pests and microbial contamination. This can be particularly advantageous e.g. in the dissemination of the germplasm. A further advantage is that relatively little space is needed to preserve large amounts of plant germplasm and require less maintenance (Uyoh *et al.*, 2003).

Medium–term storage

Medium–term storage techniques have been developed for a large variety of plant species, and are now routinely used for the genetic resources preservation of species such as banana, potato or cassava (Engelmann, 1997a). The primary aim of medium-term storage is to minimize growth and to increase the intervals between subcultures (Engelmann, 1999). Medium-term storage is commonly accomplished by decreasing culture temperature (Engelmann, 1998). Tissues or cells can also be placed into a state of slow growth in the presence of growth regulators (natural or synthetic) or osmotically active compounds (Withers, 1987a, 1987b). Selected *Eucalyptus* types can be successfully stored for periods of six to ten months using this technique (Watt *et al*. 2000a; 2000b).

Long-term storage

Long-term storage of plant germplasm is achieved via cryopreservation, where plant tissues and cells are stored at ultra low temperatures (Benson *et al.,* 2006; Rao, 2004; Wang, *et al*., 2002). Long-term storage (cryopreservation) is usually at temperatures below -130˚C (-202˚F), with liquid nitrogen being the most commonly used cryogen (-196˚C/-320˚F) (Touchell and Dixon, 1999). Storage at these low temperatures slows down or stops metabolic processes (Engelmann, 1997a; Rao, 2004) and hence allows for the protracted storage of the material with the minimum of genetic change.

Cryopreservation

The cryopreservation method guarantees the safe storage of plant material. Furthermore only a small quantity of material is preserved and thereby protected from contamination and reducing the costs that are associated with other techniques (Engelmann, 1997a; Engelmann, 2004). The genetic structure of cryopreserved cells or tissues is assumed to be unchanged during such storage and genetic drift is thus minimized (Withers, 1980, 1982, 1985; Engelmann, 1997b). Cryopreservation of callus cells may be challenging because of the relative volume of the callus cells or tissues which may be at high water contents and their possibly slow rate of growth and their cellular heterogeneity (Withers, 1987b).

Cryopreservation procedures generally comprise of the following stages: pregrowth; drying; cryoprotection; freezing; storage; thawing and recovery (Withers, 1988).

i. Pregrowth and cryoprotection

Plant cells and tissues can be pretreated with different cryoprotectants or additives before the freezing step. Cryoprotectants are defined as compounds or groups of substances that form an amorphous glassy phase of the cellular matrix/cytoplasm by decreasing the water content of the plant material to a point that will prevent or reduce ice crystallization or may

function differently in plant cells by protecting the cells from drying and freezing damage (Kartha, 1985; Withers, 1988; Benson, 1994; Crowe *et al.*, 1998; Touchell and Dixon, 1999; Bryant *et al*., 2001; Sakai, 2004; Verleysen *et al*., 2004; Benson *et al*., 2006).

The most commonly used cryoprotectants include dimethylsulfoxide (DMSO), ethylene glycol (EG), polyethylene glycol (PEG), glycerol, sucrose, sorbitol and mannitol (Touchell and Dixon, 1999). Sucrose application to plant tissue has been shown to protect meristem cells from dehydration and ice crystal damage throughout the cryopreservation process (Dumet *et al.,* 1993; 2000). Pre-treatment with sucrose acts by decreasing the amount of water in the plant cells that is likely to form ice (Panis and Thinh, 2001). As a result, the plasma membrane remains intact and is protected against freeze induced ice damage (Panis *et al*., 1996, Thomashow, 1999). However, sucrose can be lethal in high concentrations in certain species, e.g. to *Citrus* apices (Bouman and De Klerk, 1990). Some cryoprotectants penetrate intracellularly (e.g. DMSO) whilst other cryoprotectants cannot enter cells but penetrate tissues between the cells (such as sucrose). However both types are crucial factors in minimizing the damage by ice during freezing (Towill, 1991; Benson *et al*., 2006).

ii. Drying

Water forms part of every living organism (Muldrew *et al*., 2004). Primarily plant cells contain about 95% water (Reinhoud *et al*., 2000). Water structure can be classified as gas, liquid, solid and vitreous phase (Benson *et al*., 2006). The vitreous phase can be achieved by using solutions such as cryoprotectants (Fahy *et al*., 1984; Dereuddre and Kaminski, 1992; Sakai, 2004). Water in the liquid phase freezes at 0° C at normal pressure and boils at 100° C (Benson *et al.*, 2006). When liquid water turns into ice it is called the crystalline state or solid phase (Sakai, 2004). However, water in the solid phase can also be vitreous, in which case the water enters a glassy phase rather than the crystalline state (Taylor *et al*., 2004; Sakai, 2004; Benson *et al.*, 2006).

Plant material can be chemically desiccated by treating it with cryoprotectants or osmotically active substances (Engelmann, 1997b). The material can also be first embedded in calcium alginate beads, then treated chemically with a high concentration of cryoprotectants (such as sucrose), dried, then rapidly frozen (Benson, 1994, Engelmann, 1998; Panis and Lambardi, 2005). Physical drying can be carried out over activated silica gel (Uragami *et al.*, 1990; Benson 1994; Engelmann, 1998), or drying of the material in dry air of the laminar flow (Panis and Lambardi, 2005). Other methods include forced air-drying or drying to equilibrium at low relative humidity (Groll *et al*., 2002). The drying of plant tissue essentially reduces the risk of damage by intracellular ice crystals during freezing and thawing (Reinhoud *et al*., 2000).

iii. Freezing

Plant material can be frozen in a fast, slow or stepwise manner (Kartha, 1982). In rapid freezing, samples are directly immersed into liquid nitrogen (Kartha, 1987; Benson, 1994). For many protocols, the slow or step-wise freezing requires cooling the material to low temperatures at controlled rates (between 0.5 and 2 °C/min) to fixed temperatures (-20º to/or -40º C), for specified periods, then rapidly freezing the material in liquid nitrogen (Kartha, 1982, 1987; Benson, 1994; Engelmann, 1998; Touchell and Dixon, 1999; Nsabimana *et al*., 2003; Engelmann, 2004; Rao, 2004). The latter approaches are preferably used for callus and cell suspensions (Withers, 1985; Kartha and Engelmann, 1994; Withers and Engelmann, 1998). The slow freezing process involves removal or reduction of intracellular water out of the cells at the decreased temperatures (Benson, 1994; Engelmann, 1997b). The slow freezing method is presumed to be most effective in that the cells are less prone to damage and intracellular ice formation when subsequently submerged in the liquid nitrogen (Kartha, 1987; Engelmann, 1997b).

iv. Storage

Cryostorage of material is usually in cryovials, or any other similar plastic container (Pence, 1995). Levels of liquid nitrogen are frequently monitored (Benson, 1994).

v. Thawing

Rapid thawing is mostly utilized for plant tissues as this method avoids ice recrystallization damage as opposed to slow thawing (Mazur, 1984; Withers, 1988; Gnanapragasam and Vasil, 1992). This is achieved by immersion of the cryovials or containers in a water bath at 40 ºC for one to two minutes (Pence, 1995). Slow thawing because the time to develop ice crystal is immediately passed.

vi. Recovery

Usually, after thawing, a viability test is carried out. This test estimates the effect of freezing on the viability of the plant tissue (Towill, 1991; Verleysen *et al*., 2004). Cell or tissue viability can be estimated by the capacity of the material to reduce 2,3,5, triphenyltetrazolium chloride, in which cells that are viable will stain red or pink (Towill and Mazur, 1974; Pellet and Heleba, 1998). The pathway of triphenyltetrazolium chloride is based on the enzymatic action of active dehydrogenase in mitochondria of viable plant cells. The colourless triphenyltetrazolium chloride is reduced to red (Steponkus and Lanphear 1967; Towill and Mazur 1974).

Other methods include the quantitative assay of MDA (Malondialdehyde) and MEL (Meristem electrolyte leakage) (Verleysen *et al*., 2004). MDA is formed during lipid peroxidation due to injury to membranes and to a certain extent, to cell walls (Harding and Benson, 1995). Injury to the cell membrane due to intracellular ice crystals leads to cell leakage (Ketchie *et al*., 1992; Grout, 1991a,1991b; Mazur, 2004). Most importantly the recovery must also be assessed by regrowth, which determines the capability of the plant material to regenerate and multiply after freezing (Grout, 1995). For conservation purposes the regrowth test is definitive.

1.3.3 Genetic modification of forest trees

Genetic modification is an important tool in forestry as it allows for the introduction of specific traits to selected clones (Ahuja, 2000). Through the use of genetic engineering on forest plantation species, the quality of wood can be improved, new industrial processes, such as new enzymatic processes for lignin degradation can be developed, pesticides and disease tolerant trees can created and the rooting ability for hard-to-root species can be enhanced (Strauss *et al*., 1995; Meilan, 2001; Chiang, 2002; Pilate *et al*., 2002; Campbell *et al.*, 2003; Mashkina and Butorina, 2003; Hoenicka and Fladung, 2006; Pijut *et al*., 2007). Thus far the genetic transformation of forest trees has been accomplished with two technologies *viz*. *Agrobacterium tumefaciens* and the gene gun (Pijut *et al*., 2007).

The success of genetically modified forest species can be limited because of insufficient propagation techniques exist for the successful multiplication of transformed clones, field trials performance and commercially deploying them (Balocchi and Valenzuela, 2004). For hardwoods such as *Eucalyptus* the main genetic engineering approach employs *Agrobacterium*-mediated transformation and the regeneration of plants via indirect organogenesis and this has been successfully implemented in some *Eucalyptus* clones e.g. *E. globulus* (Moralejo *et al*., 1998) and *E. camaldulensis* (Ho *et al*., 1998).

1.4 AIMS

In previous studies in our laboratories somatic embryos and axillary buds (Padayachee *et al*., 2008) have proven unsuitable propagules for cryopreservation of *Eucalyptus.* In this study an alternative approach was used in an attempt to cryostore callus initiated from axillary buds via an indirect organogenesis procedure. Callus was chosen as the cryopreservation material because it contains single totipotent cells and a regeneration protocol to induce shoots and roots is available (Hajari *et al*., 2006). Previous studies (e.g. Stewart *et al*., 2001) have shown that explant developmental status is important for cryopreservation success. It is therefore decided to utilise microscopy to describe the development of the structures within the callus and use that information to determine an appropriate developmental stage for cryostorage.

Towards this end the specific aims of this study were to:

(1) identify cells responsible for the initiation and development of callus from axillary buds and describe the morphology of the callus in terms of colour and texture;

(2) identify cells responsible for the initiation and development of shoot-buds regenerated from the callus;

(3) identify cells responsible for the initiation and development of roots formed from the shoots developed from the callus;

(4) develop a cryopreservation protocol for the callus.

2. MATERIALS AND METHODS

2.1 Callus initiation, shoot and root organogenesis (Figs. 1.1; 1.2)

2.1.1 Source of explants and maintenance of material in the greenhouse

The plant material that was used in this study was a clone of *Eucalyptus grandis x urophylla* (GU 185) obtained from Trahar Laboratory, Mondi Business Paper, Hilton, KwaZulu-Natal, South Africa. The plants were maintained in the greenhouse at the University of Witwatersrand, monitored everyday and given a daily supply of water and fertilizers once a week. The foliar spray fertilizer that was applied to the plants was 2.5 ml/L trace element solution (18 g/L Fe, 4 g/L Cu, 2 g/L Zn, 1 g/L B and 0.4 g/L Mo) and Mondi orange IN-2P-1K (1 g/L) was applied as a soil spray. Fungicides were applied every week (once) to protect the plants against harmful fungi. The fungicide foliar spray contained a mixture of 2 g/L mancozeb and 1 ml/L of chlorothalonil. Fungicides that were applied to the soil contained a mixture of 1 g/L of prochloraz manganese chloride and 1.25 ml/L tebuconazole.

2.1.2 Preparation and multiplication of shoots under sterile conditions

Nodal shoot sections (80-90 mm in length) were excised and surface decontaminated in a solution of 0.2 g/L $HgCl_2$ containing one drop of Tween 20 for ten minutes and rinsed three times in sterile water. The nodal segments were then placed in a solution of 0.2 g/L $Ca(OCl)_2$ for ten minutes and rinsed again three times in sterile water. They were then placed in a mixture of 50 mg/l of penicillin and 50 mg/l of streptomycin for ten minutes, trimmed to two-node shoots (30-40 mm) and plated on multiplication medium [MS salts and vitamins (Murashige and Skoog, 1962), 25 g/L sucrose, 0.1 mg/L biotin, 0.1 mg/L calcium pantothenate, 0.01 mg/L NAA (α-napthaleneacetic acid), 0.2 mg/L 6- BAP (benzylaminopurine), 2.3 g/L gelrite, and the pH of the medium was between 5.6 and 5.8]. The medium was autoclaved for 20 minutes, at 120 ºC and pressure of 121 kPa. The material was incubated in a growth room (under 16-hour photoperiod, PPFD

(photosynthetic photon flux density) of 37 µmol m^{-2} s^{-1}, at 25 ºC day/21 ºC night. The new shoots that formed were removed, separated and subcultured every four weeks.

2.1.3 Initiation of callus and regeneration of shoots

The method of Hajari *et al.* (2006) was used. Axillary buds were isolated from the subcultured shoots and cut into small segments (0.5 mm-1 mm) with a sterile blade i.e. one axillary buds was cut into two or three segments. The pieces of axillary buds were plated (100-120 pieces per 90 mm Petri dish) on the callus induction medium (20ml per Petri dish): full strength MS medium salts with vitamins (Murashige and Skoog, 1962), 30 g/L sucrose, 4 g/L Gelrite and 5 mg/L IAA (indole-3-acetic acid) and 0.25 mg/L BAP (6-benzylaminopurine), pH of medium between 5.6 and 5.8. The material was cultured in the dark for four weeks, at 24 to 26 ºC, and the callus was transferred to fresh medium and any regenerated shoots were removed from the callus. Callus was then incubated for a further four weeks in constant light. The callus produced shoots after four weeks of subculture in callus induction medium. The material was transferred on semi-solid medium [MS salts and vitamins (Murashige and Skoog, 1962)], 25 g/L sucrose, 0.1 mg/L biotin, 0.1 mg/L calcium pantothenate, 0.01 mg/L NAA (α-napthaleneacetic acid), 0.2 mg/L 6- BAP (benzylaminopurine), 2.3 g/L gelrite, pH of the medium between 5.6 and 5.8 for a further four to five weeks.

2.1.4. Rooting of shoots

The shoots were removed from the callus, individually separated and transferred onto a hormone free medium: full strength MS salts and vitamins (Murashige and Skoog, 1962), 15 g/L sucrose and 4 g/L Gelrite, for one week, then rooted in rooting medium (¼ MS salts and vitamins, 15 g/L sucrose, 0.1 mg/L biotin, 0.1 mg/L calcium pantothenate, 4 g/L gelrite, 0-1 mg/L IBA (Indoleacetic acid) , with the pH of the medium between 5.6 and 5.8, at 24 to 26 ºC, for four weeks. The shoots were first rooted in the dark for 72 h, then exposed to a 16-hour photoperiod, PPFD of 37 µmol m^{-2} s^{-1} (overhead

lighting) at 24 ºC day/26 ºC night. Each culture vessel contained 20 ml of rooting medium with two to three shoots.

2.1.5. Acclimatisation of regenerated plantlets

The rooted plantlets were acclimatised in a two-step process, firstly in rooting mix then in autoclaved sand. The rooting mix contained perlite and coir (2 perlite: 1 coir). The plantlets were first rinsed in sterile water to remove the medium from the roots and potted in rooting mix. The plants were soaked with 1/3 MS salts and covered in large plastic bags (120 x 100 cm). The plants were placed in the growth room under standard conditions (see earlier). The humidity was reduced after two weeks by creating holes in the plastic bags. By the fourth week, the plants were transferred into autoclaved sand supplied with 1/3 MS salts for a further four weeks and planted in black plastic bags (17 x 14 cm) then transferred to the greenhouse.

2.1.6. Microscopy

Light microscopy was used to assess callus, shoot organogenesis, callus-shoot vascular connection and shoot-root vascular connections. Samples of callus (0.1-0.2 mm) were initially taken after 15 days during the eight weeks required for callus and shoot induction, thereafter every seven days for the entire developmental process. The roots samples (1-5mm in length) were taken on day one and day three while on the rooting medium. Samples were sectioned through entirely, particular care was taken when checking for the vascular connections with each section being collected and viewed**.** The samples were fixed in a solution (pH 7.2) of 3% glutaraldehyde and 0.1 M phosphate buffer [2ml of 25% glutaraldehyde (25/100ml) and 88ml of phosphate buffer] for 24 hours at room temperature. The phosphate buffer solution was a mixture of 72ml disodium hydrogen orthophosphate (14.2 g/L) and 28ml of orthophosphate and sodium dihydrogen orthophosphate dihydrate (15.6 g/L) pH 7.2. The samples were then washed in phosphate buffer by rinsing every 10 minutes an hour, then post-fixed in I vial of 0.5% osmium tetroxide (5/100ml) with an equal volume of the 0.1 M phosphate

buffer prepared above for an hour in the fume cupboard. The samples were dehydrated through 10% ethanol (10ml/100ml), 25% ethanol (25ml/100ml) and lastly 50% ethanol (50ml/100ml). The samples were placed in uranyl acetate solution (25g of uranyl acetate salt in 25ml of 75 %ethanol) for an hour and dehydrated further in 70% and 100% ethanol. The samples were polymerized in epoxy resin (Spurr, 1969) in an oven for eight hours at 80° C. The epoxy resin embedding medium components were Vinylcyclohexene (VCD), hardener:nonelyl succinic anhydride (NSA), plasticiser:DER 736 and accelerator:dimethylaminoethanol (DMAE). DMAE was the last component to be added to the mixture. An ultra microtome (Reichert-Jung Ultracut) was used to cut sections of 1 µm in thickness which were stained with toulidine blue solution [60 ml sodium bicarbonate (1g/100ml water), mixed with 40 ml glycerol and 1g toulidine blue) and mounted on glass slides using DPX (di-n-butylphthalate in xylene). The sections were viewed and photographed with the light microscope (Zeiss Axiophot photomicroscope). Fifteen samples of each developmental stage were viewed.

2.2 Callus cryopreservation (Fig. 1.3)

2.2.1. Drying and cyoprotection of callus

In this study, 22 day old callus was cryopreserved. The pieces of callus (<2 mm in diameter) were dried in a dessicator (4500cm^3) over activated silica gel (330 g) for 20 minutes. The pieces of callus were dried either prior to or after treatment with cryoprotectants. The cryoprotectant treatment involved exposing callus to 5% (v/v) dimethysulfoxide (DMSO) containing 5g sucrose for 15 minutes, blotted on sterile paper to remove excess solution. The callus was submerged in 10% (v/v) Dimethysulfoxide (DMSO) containing 10 g sucrose for 15 minutes. After exposure excess solution from samples was removed by blotting on sterile paper.

2.2.2. Viability test after drying and treatment with cryoprotectants

Viability after drying was determined by exposing the callus to 2,3,5 triphenyltetrazolium chloride (0.01g in 100ml sterile water) in dark conditions for 24 hours. Survival was also assessed quantitatively by observing the ability of the callus to undergo morphogenesis.

2.2.3. Water content

Water concentration was determined gravimetrically after incubating callus explants at 80 ºC for 24 hours and expressed on a wet weight basis. Water concentration was determined both before and after drying over silica gel and after treatment with the cryoprotectants.

2.2.4. Freezing and thawing of callus

The callus pieces were frozen using two different freezing regimes, slow and rapid. The slow freezing process was a two-step procedure; callus pieces were placed in cryovials (5 pieces per vial) and frozen in a Mr Frosty (Sigma) unit, containing 250 ml of isopropanol, to – 70° C for an hour, then the cryovials were fixed on cryocanes and immersed into liquid nitrogen at -196° C for 45 minutes. The rapid freezing involved only one step freezing of callus in cryovials fixed to cryocanes in liquid nitrogen at -196° C for 45 minutes. The cryocanes were thawed in a water bath at 37 ºC for one to two minutes for both freezing methods.

2.2.5. Recovery after freezing

The viability of the callus was determined after freezing by incubating the callus in 0.01% (v/v) of 2,3,5 triphenyltetrazolium chloride (0.01g in 100ml sterile water) in dark conditions for 24 hours (ISTA, 2003). The survival of callus was also determined by monitoring the ability to undergo morphogenesis on callus induction medium (2.1.3) for four weeks.

2.2.6 Statistics

Each treatment was repeated at least three times per treatment (20 samples per treatment) and the mean and standard deviation was determined for each result. A T-test was used to assess differences between the treatments.

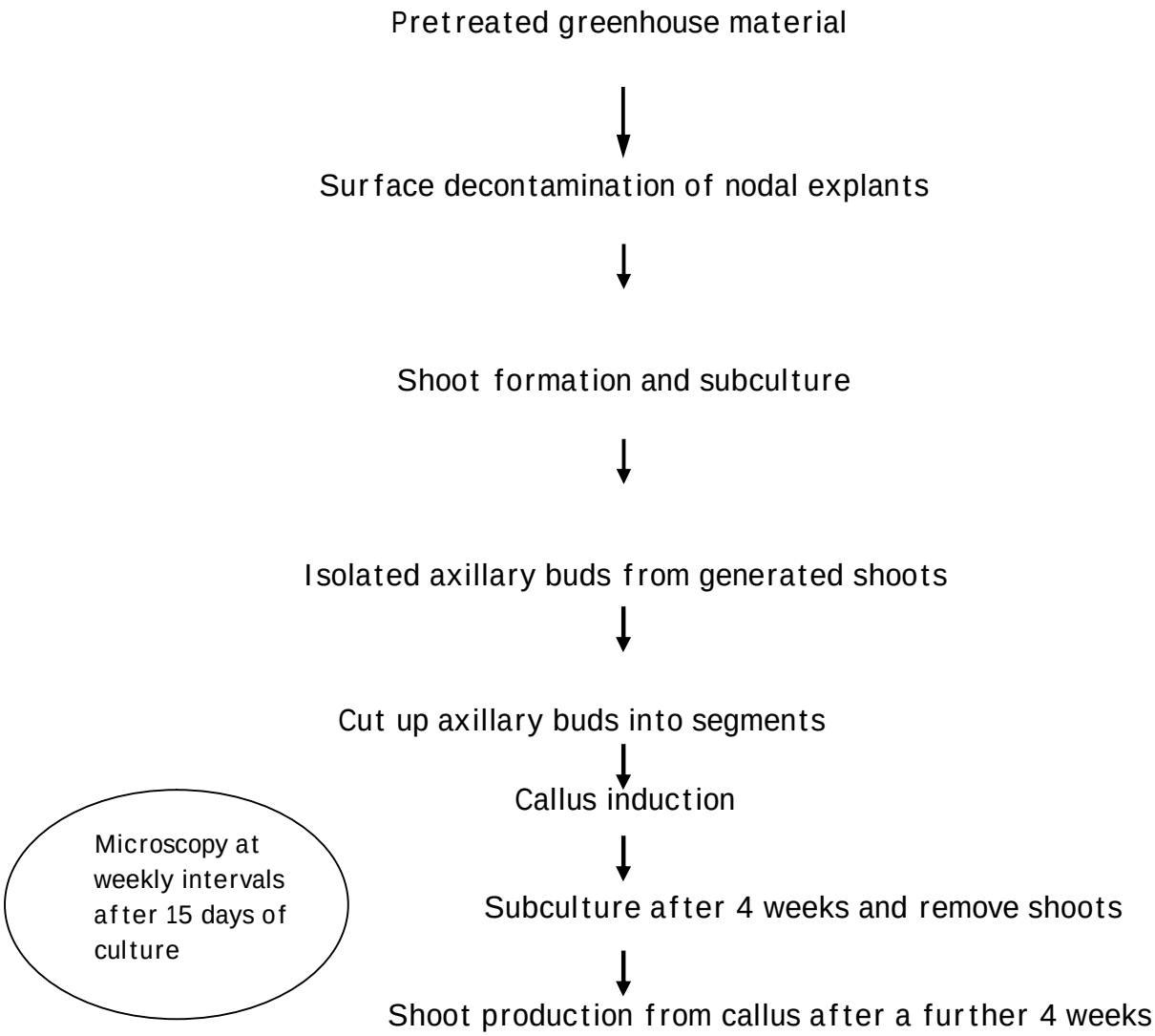


Figure 1.1 Schematic representation of regeneration of adventitious shoots from callus *of E. grandis* x *urophylla in vitro.*

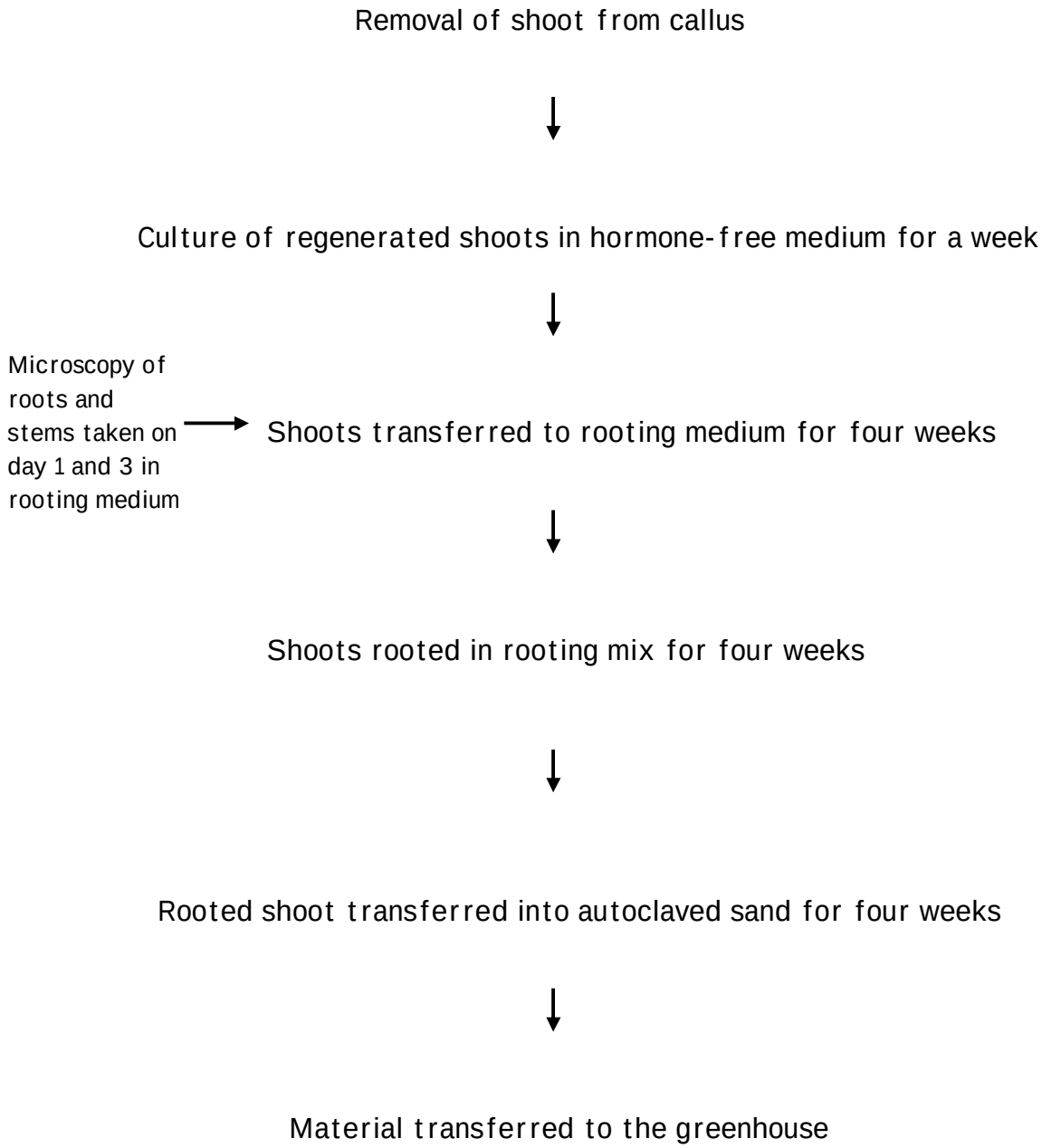


Figure 1.2 Schematic representation of rooting procedure in of *E. grandis x urophylla in vitro*.

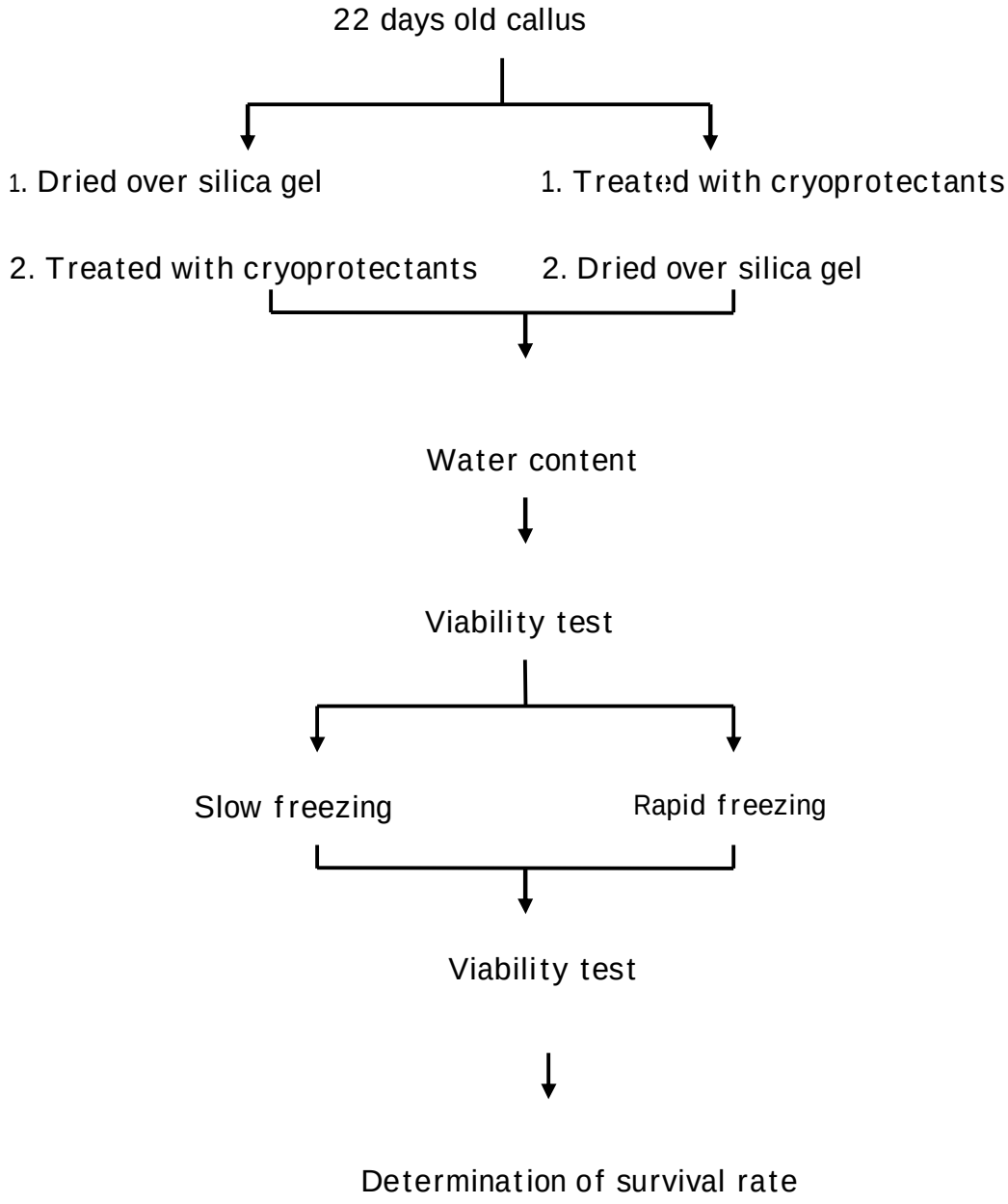


Figure 1.3 Schematic representation of freezing of *E. grandis x urophylla* callus.

3. RESULTS

3.1 Callus induction and shoot-bud development

Eucalyptus plants that were maintained in the greenhouse were used as a source for shoot regeneration and production. The nodal shoots with two buds each were removed from the plants, decontaminated and multiplied on semi-solid multiplication medium for generation of *in vitro* shoots (Figs 2A and 2B). By the end of experimentation, 86% of the explants produced callus, but only 39% of these developed shoots.

Table 2: Callus and shoot formation from *Eucalyptus grandis x urophylla* in vitro axillary buds after eight weeks of culture. n=100-120, ± SD.

Age in Days	Callus appearance	Shoots	% pieces of segments from individual bud that formed callus	% Callus with shoots
15	Crystalline,Compact (white,yellow and brown).	NS	73 ± 9	0
22	Crystalline,Compact (white,yellow and brown), Nodular.	S	84 ± 12	2 ± 3
29	Crystalline,Compact (white,yellow and brown), Nodular.	S	86 ± 11	11 ± 1
36	Crystalline,Compact (white,yellow and brown), Nodular.	S	86 ± 11	27 ± 6
43	Crystalline,Compact (white,yellow and brown), Nodular, Red.	S	86 ± 11	28 ± 7
50-57	Crystalline,Compact (white,yellow and brown), Nodular, Red.	S	86 ± 11	39 ± 6

(NS-No shoot formation, S-Shoot formation, vascular tissue formed in all the stages).

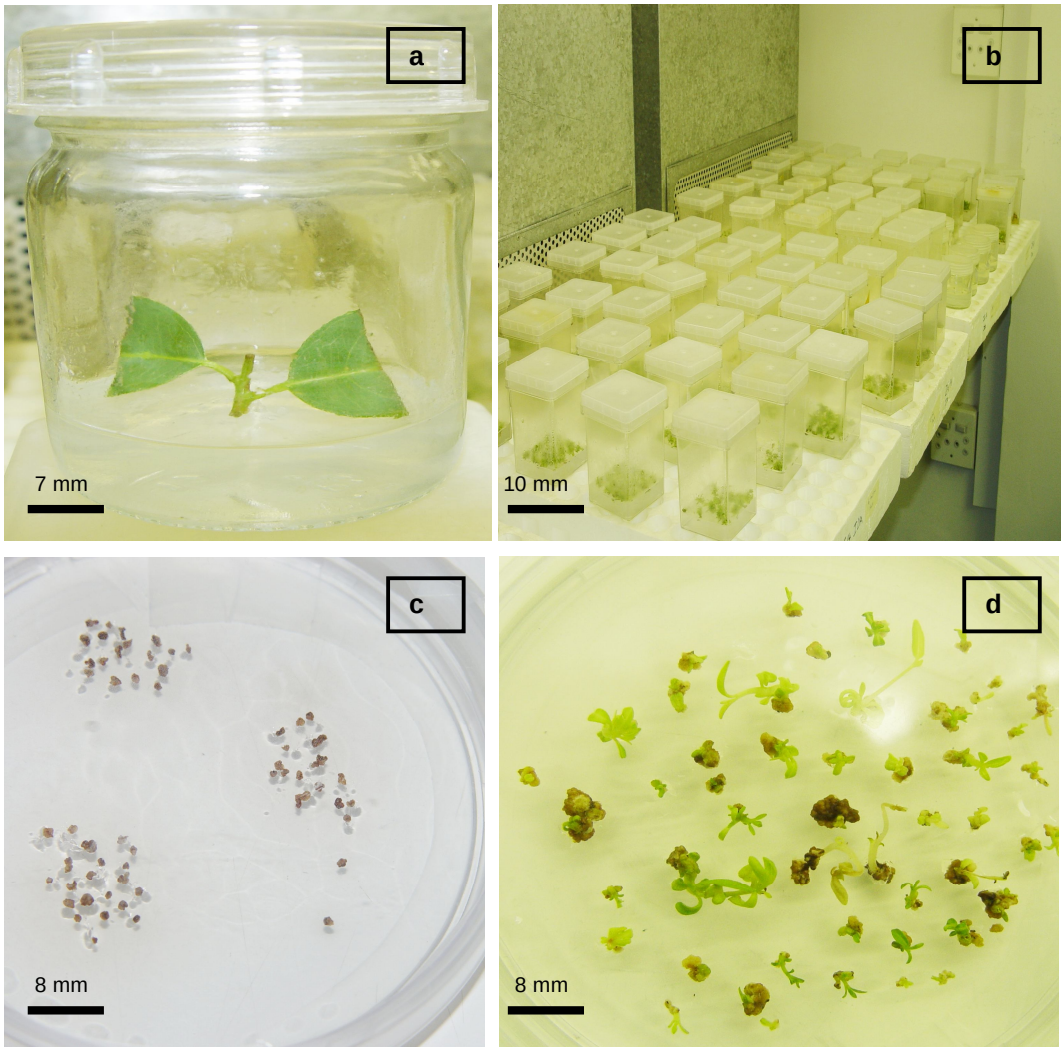


Figure 2: Cultures exhibiting the various stages of indirect organogenesis (callus initiation and shoot organogenesis) in *Eucalyptus grandis x urophylla*. a: Shoot with two nodes after being surface decontaminated and cultured on semi-solid medium. b: Shoot multiplication in the growth chamber. c: Initiation of callus. d: Shoot-bud development from callus pieces.

3.1.1 Callus morphology 15 days after callus initiation on induction medium

Callus formation was visible within 15 days of induction. At this point only 73% of cultured explant (one axillary bud cut into small segments) had formed callus (Table 2). The different types of calli were characterised according to their structure, texture and type of component cells. The callus of this age included those with a crystalline surface (Figs. 3b, f), a shiny or glassy surface which appeared brown, yellow or white (Figs. 3a,b,c,d,e) and calli that had dark patches (Figs. 3a,d). Anatomically the calli consisted of different types of cells and tissues, predominantly morphogenic cells (Figs. 4b,f; 5e) nonmorphogenic cells (Figs. 4b,e,f; 5d,e), and vascular tissue (Figs. 4c,5c,d,e). In the context of this study the term morphogenic cells describes a group of cells which appear meristematic- like which are tightly packed with each cell containing a clearly visible central nucleus. Large irregular shaped cells with a small nucleus and a relatively large cytoplasm when compared with the morphogenic cells are described as nonmorphogenic cells. These cells were either situated on the surface of the callus or were deeper in the callus mass surrounded by morphogenic cells.

Nonmorphogenic cells that were formed on the surface of the callus were irregular in size and shape when compared with morphogenic cells which were smaller and packed together (Figs. 4b, e, f; 5d, e). Some of the cells were stained darkly with the toulidine blue, and this might indicate the presence of tannins in the cells (Figs. 4d; 5d). Unusual groupings of meristematic-like cells were also recognised. These cells were packed together and there was a prominent gap in the middle of the tissues thus formed (Fig. 4b, c, e). Closer examination of the morphogenic cell layers revealed that cells were tightly packed, angular and had large prominent nuclei and nucleoli (Figs. 4e,f). The cells appeared to be differentiating as evidenced by the numerous coalescing vacuoles (Figs. 4e,f). Components of vascular tissues, particularly xylem vessels were also observed in the callus (Figs. 4c,d; 5b, c,d,e). The total yield of callus formed from explants was 73%.

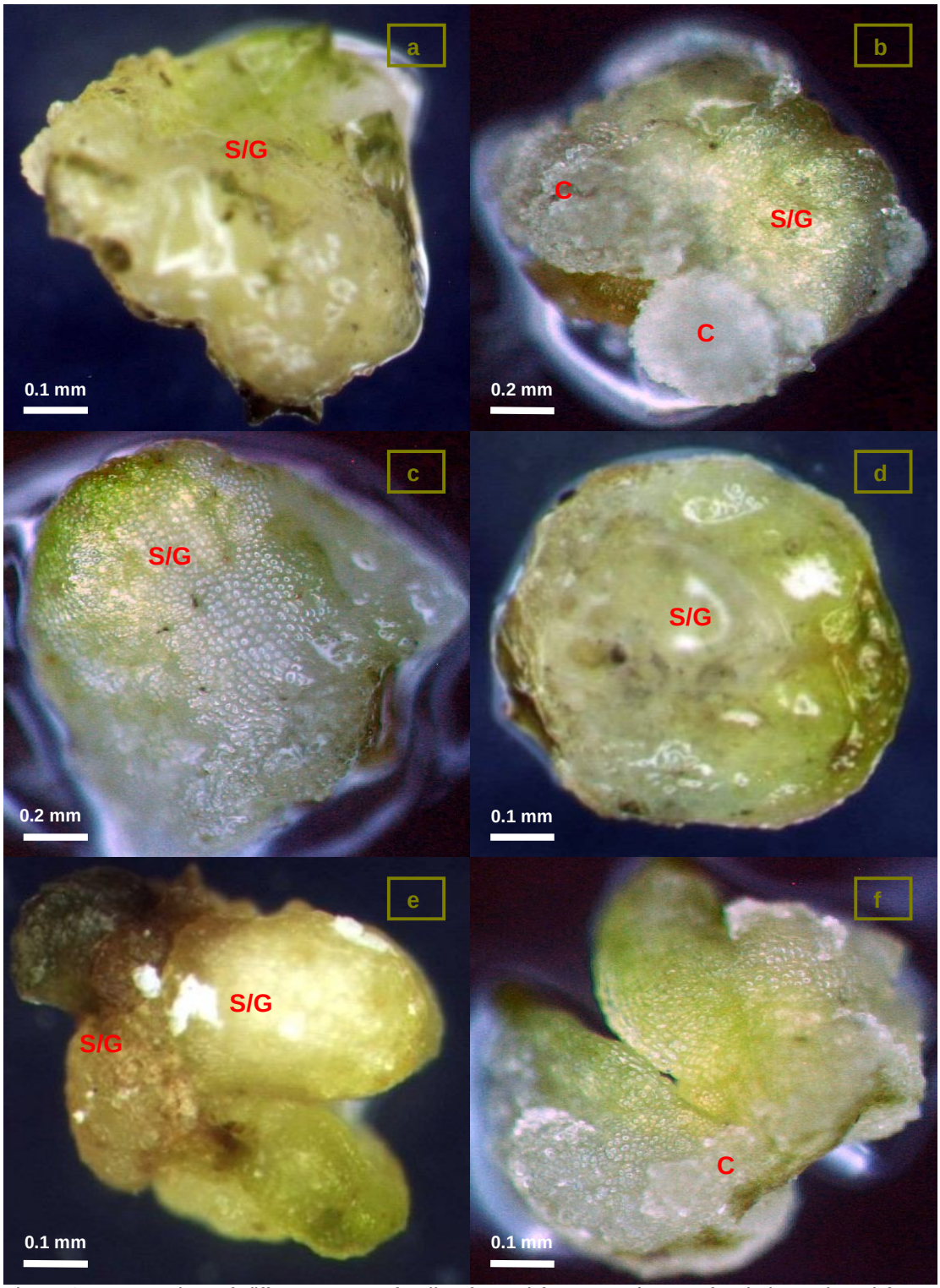


Figure 3: An overview of different types of callus formed from *Eucalyptus* after being cultured for 15 days on callus induction medium. The callus was described according to texture and colour. 3a,b,c,d,e-Shiny/glassy-brown/yellow/white), 3b, f- Crystalline callus, 3a,d- Callus with brown/dark patches on the surface, 3e,f-Callus with the remains of explant. Crystalline (C), shiny/glassy (S/G).

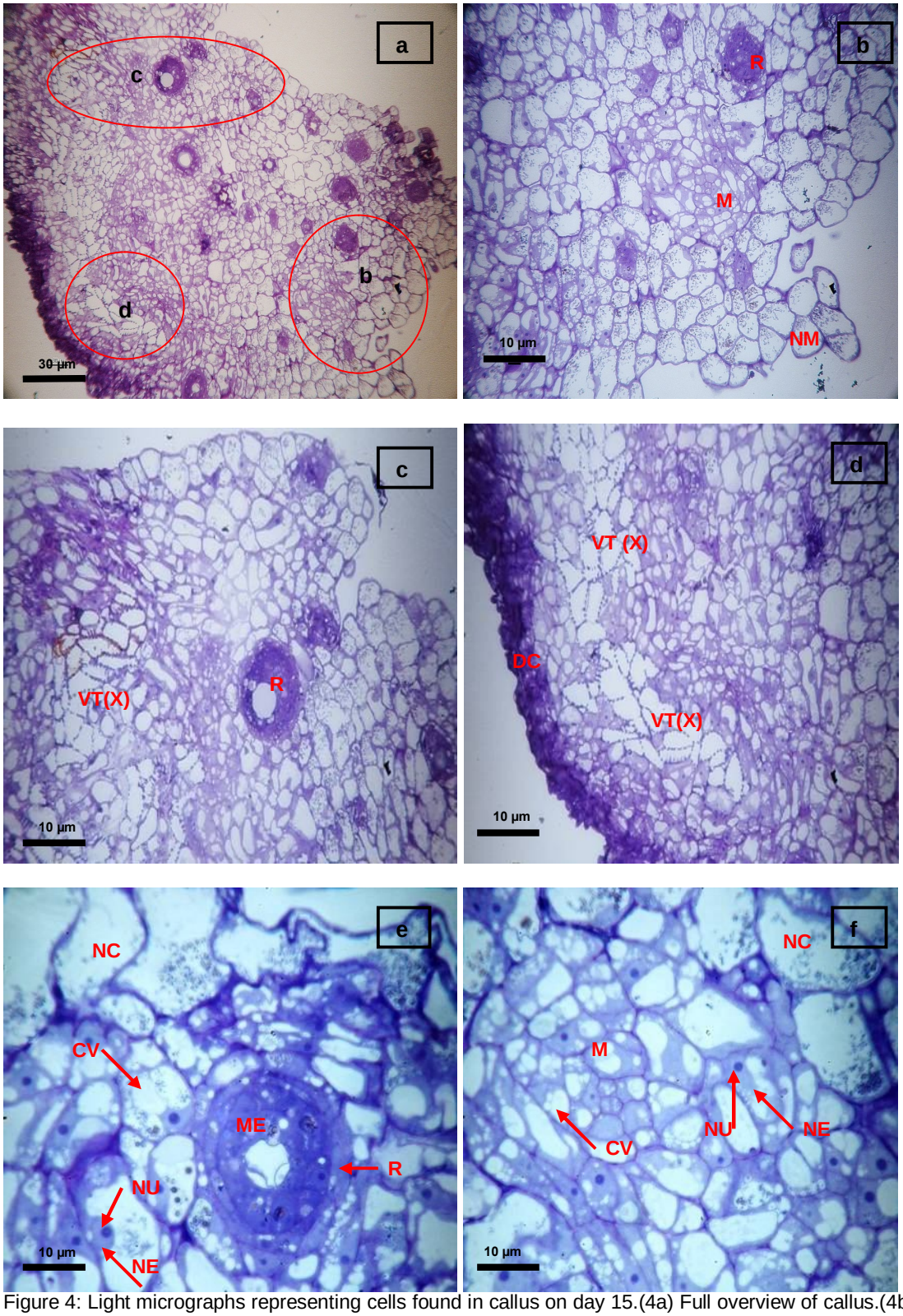


Figure 4: Light micrographs representing cells found in callus on day 15.(4a) Full overview of callus.(4b) Section showing groups of morphogenic cells (N) and nonmorphogenic cells (NC).(4c) Vascular tissue organization (VT) composed of xylem vessels (X) and rounded stacked cells (R).(4d) Dark-stained cells (DC) and vascular tissue strands (VT) with xylem (X).(4e,f) Closer observation of the meristematic regions (ME) of rounded stacked cells (R), coalescing vacuoles (CV), nuclei (NE) and nucleoli (NU),.

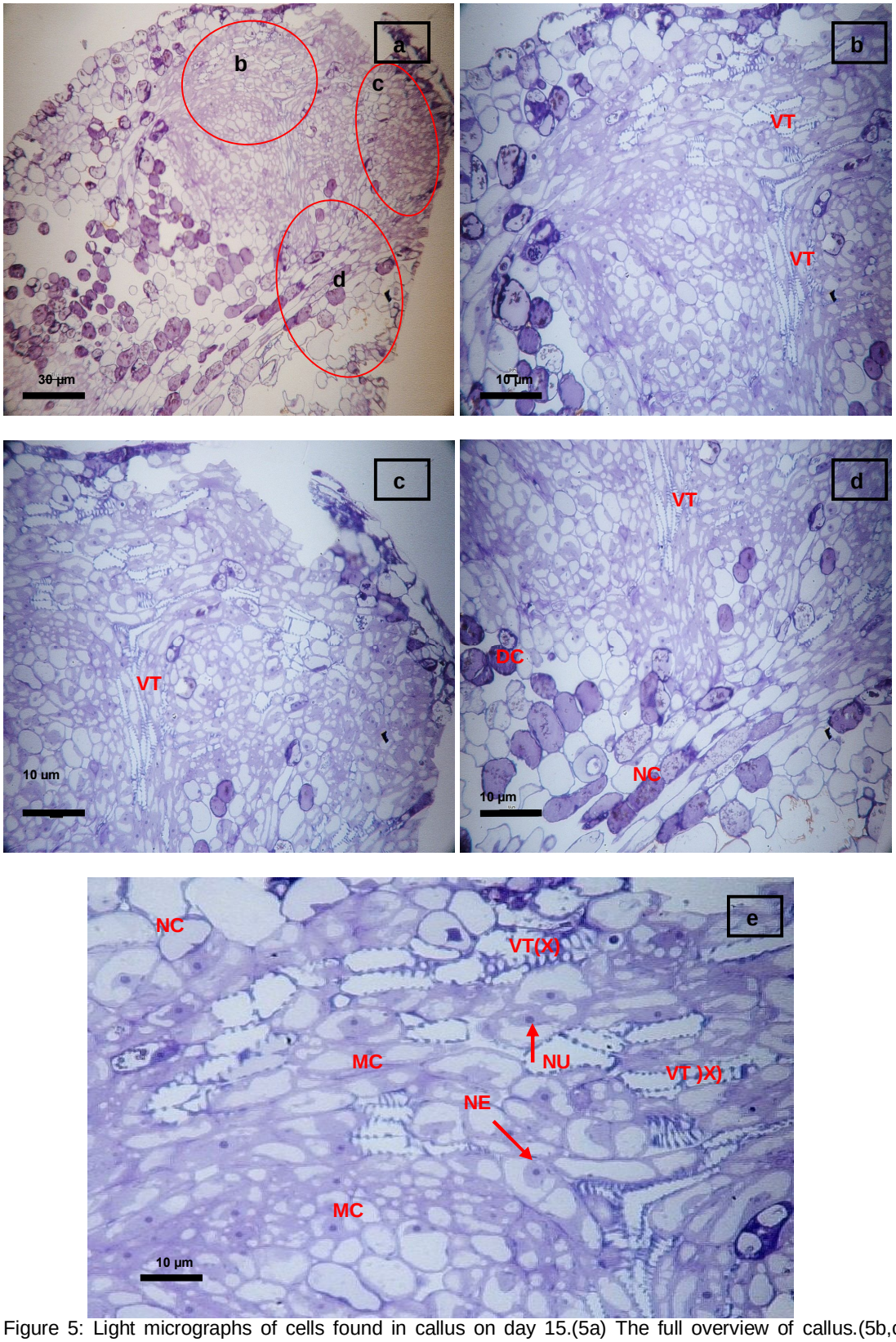


Figure 5: Light micrographs of cells found in callus on day 15.(5a) The full overview of callus.(5b,c) Strands of vascular tissue (VT) with xylem vessels (X).(5d) Dark-stained cells (DC) and large round or elongated nonmorphogenic cells (NC).(5e) Vascular tissue (VT) composed of xylem vessels (X) and morphogenic cells (MC) with visible nuclei (NE) and nucleoli (NU) and nonmophorgenic cells (NC). Note also the difference in cell size and shape.

3.1.2 Callus morphology after 22 days of culture on callus induction medium

After 22 days of culture, nodular structures started to protrude from the surface of the callus. The calli exhibited features similar to those described after 15 days of induction (Fig. 6a,b,c,d,e,f). However the crystalline layer covering the callus had increased (Figs. 6b, c, e, f). Some calli showed green areas (Figs. 6c,d,ef), which were either the remains of the explant tissue, or that represented the initiation of shoot-bud structures.

Histologically, the callus was observed to be comprise of morphogenic and nonmorphogenic cells (Figs. 7b, c, d; 8b, c, d). Morphogenic cells were small and clustered and in this instance vascular tissue (xylem vessels) formed along or next to the cells (Figs. 7c; 8b,c,d). Noticeable shoot-bud like structures formed on the surface of the callus and were covered by an apparent epidermal layer (Figs. 7a,b; 8a,b) and this can be related to the green 'callus' described in figures 6 (c,d,e,f). The cells found in the shoot-bud like structures displayed traits of meristematic cells, i.e. small, with darkly stained cytoplasm and prominent nucleoli (Figs. 7b; 8b). The epidermal covering consisted of uneven large cells, which were either rectangular or round (Figs. 7b,e; 8c). Cells that were stained darkly with toulidine blue were also visible as shown in Figures 7 and 8. The cells that were located on the periphery of the callus were large nonmorphogenic and variable in size (Figs. 7c; 8c). The yield of explants that produced callus at this stage was 84% (Table 2).

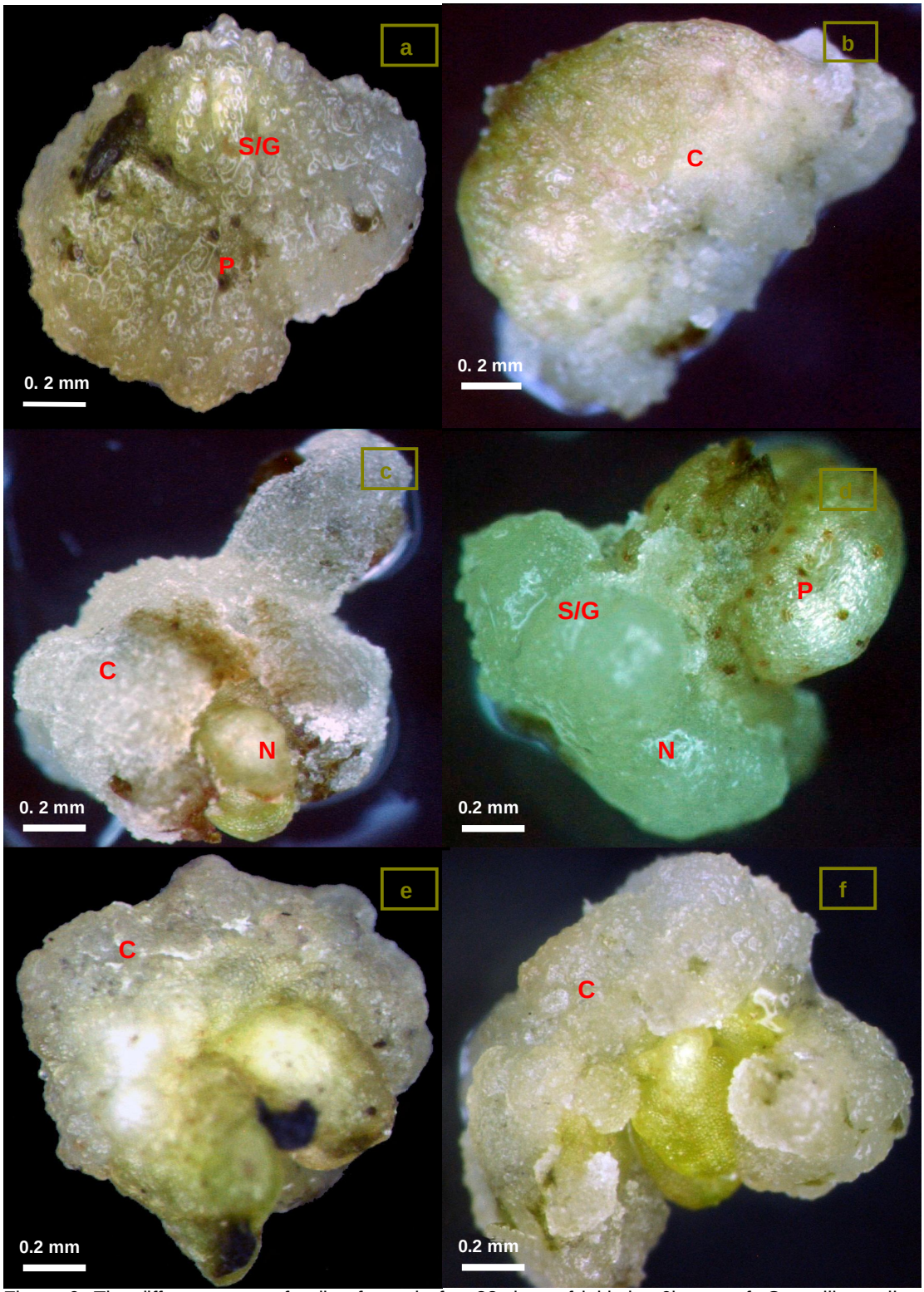
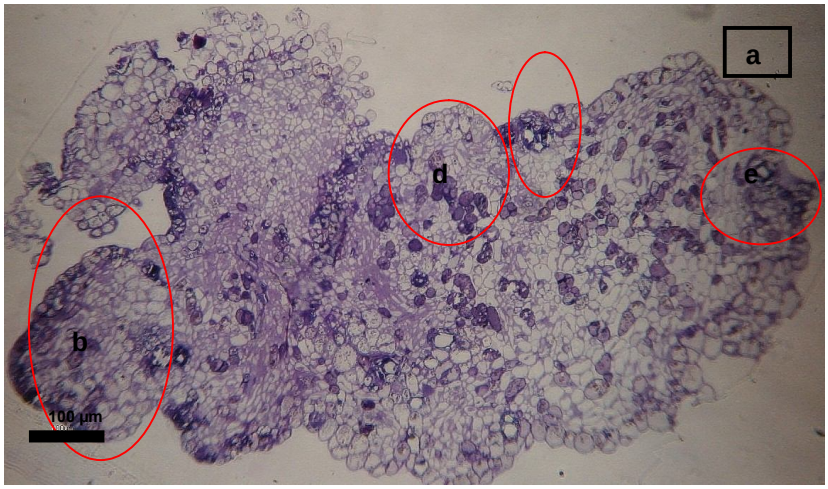
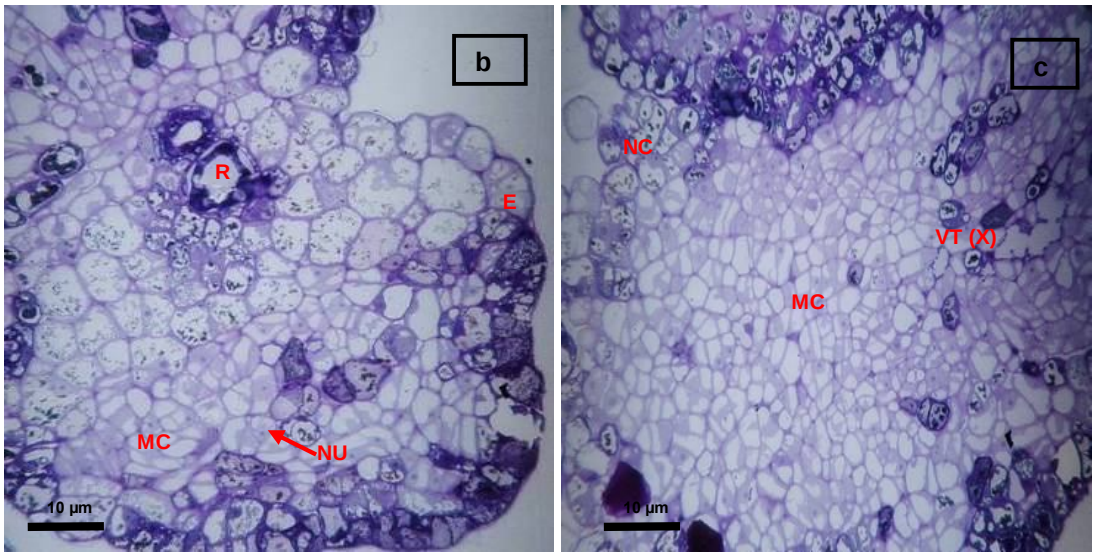


Figure 6: The different types of callus formed after 22 days of initiation.6b, c, e, f- Crystalline callus, 6a,d- Shiny/glassy and brown/yellow/white, 6a,d,c- Callus with brown/dark patches on the surface. 6c,d,e,f-Nodular callus. Crystalline (C), Shiny/glassy (S/G), Callus with dark patches (P), Nodules (N).





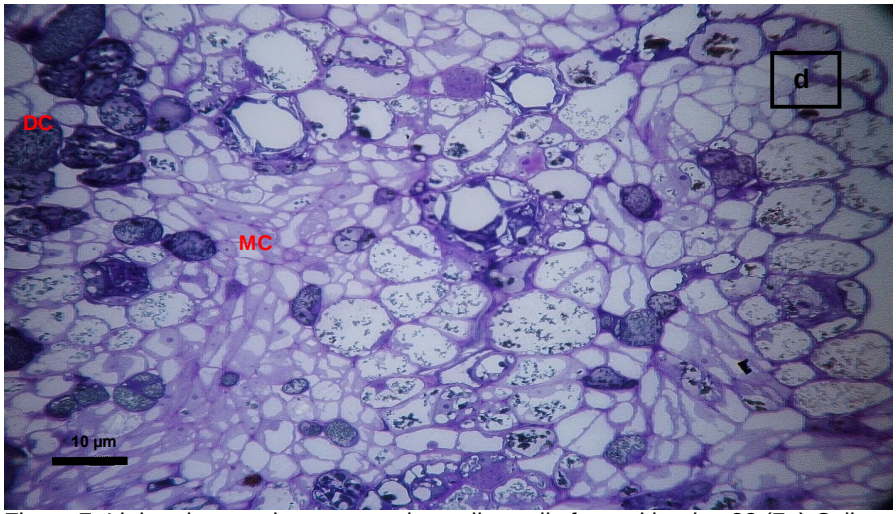


Figure 7: Light micrographs representing callus cells formed by day 22.(7a) Callus showing the initiation of a shoot-bud like structure.(7b) Closer observation of shoot-bud like nodular structure with nucleoli (NU) . Morphogenic (M), epidermal-like cells (E) and round structured cells (R).(7c) Morphogenic (M) and nonmorphogenic cells (NC) vascular tissue (VT) composed of xylem.(7d) Dark-stained cells (DC) and morphogenic cells (NC).

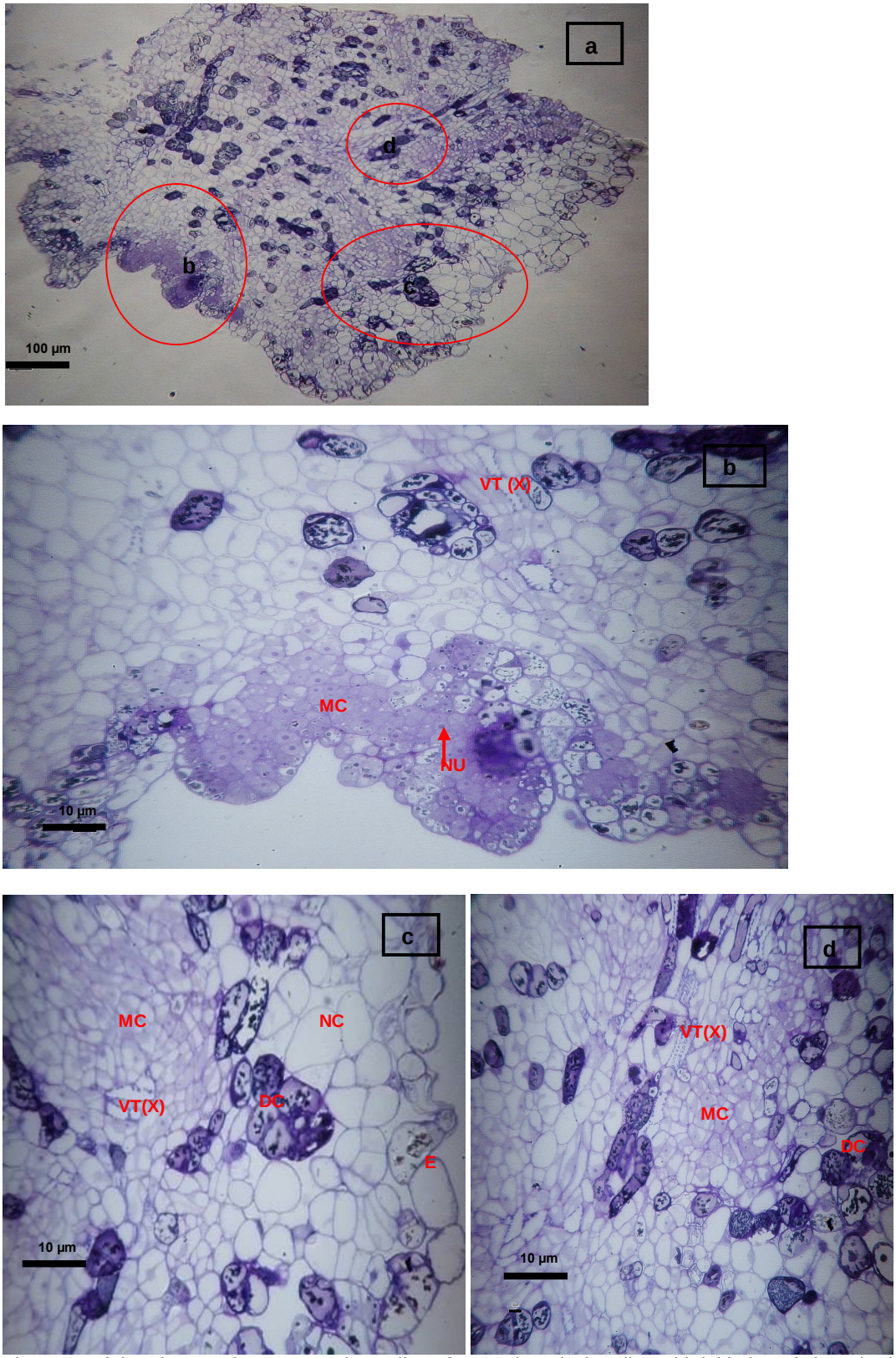


Figure 8: Light micrographs representing callus after 22 days.(8a) Callus with initiation of shoot-bud like structure.(8b) Shoot-bud like structure composed of morphogenic cells (MC) with nucleoli (NU) and traces of vascular tissue (VT) with xylem (X) .(8c,8d) Meristematic division of morphogenic cells (MC). Nonmorphogenic cells (NC) developed on surface of callus and dark-stained cells (DC). The vascular tissue was located close to morphogenic areas. Epidermal-like cells (E) formed a layer on surface of callus.

3.1.3 Developmental process of callus after 29 days on induction medium.

Twenty nine days after callus initiation the same types of calli (% of explants that produced callus was 86%) as observed on day 22 were identified, ranging from crystalline (Figs. 9a,b,c,d) to shiny/glassy callus without any crystalline layer (Figs. 9e,f). As observed at day 22, calli with green area of possible remaining tissue or developing shoot-bud like structure were also recorded (Fig. 9d).

The cells that characterized the callus were similar to those observed at days 15 and 22. However, the formation of primary vascular tissue connection between clusters of cells was a new characteristic of this stage (Fig 10). The connection formed a chain of vascular strands connecting to the morphogenic cells or morphogenic cells enclosing the vascular tissue with xylem (Figs. 10a,b,c,d,e; 11c,d,e). At each connecting point, the morphogenic cells appeared to be proliferating. Nonmorphogenic cells and darkly stained cells also formed part of the callus (Figs. 10c,e,f; 11c,d,e). The rounded structures noted on day 15 and 22 were once again identified (Fig. 11e). Epidermal-like cells developed a layer on the surface of calli (Figs. 10d; 11b). A possible developing shoot-bud was evident (Fig. 10d).

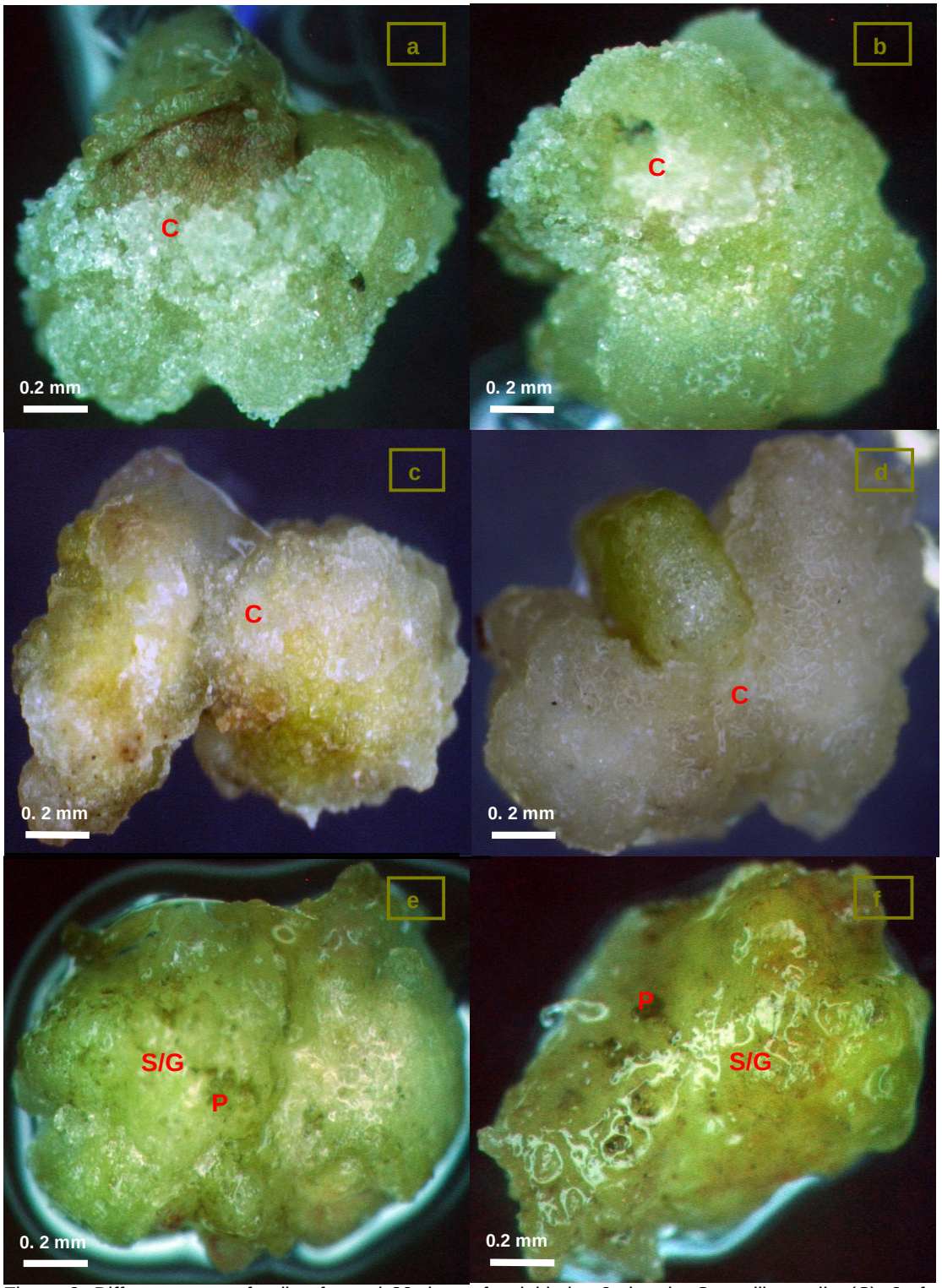
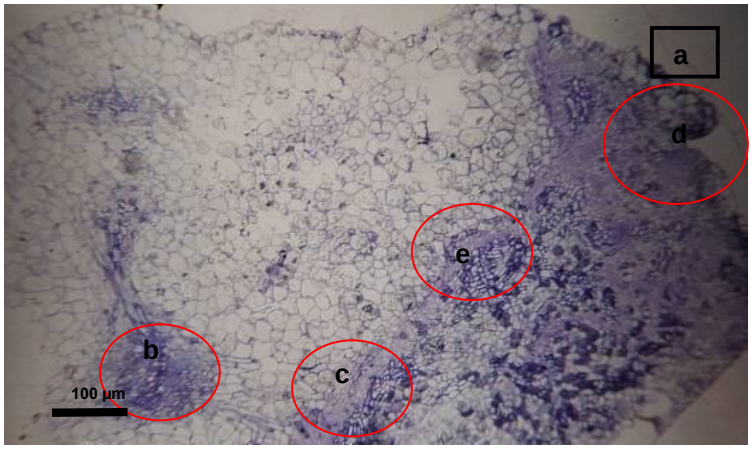
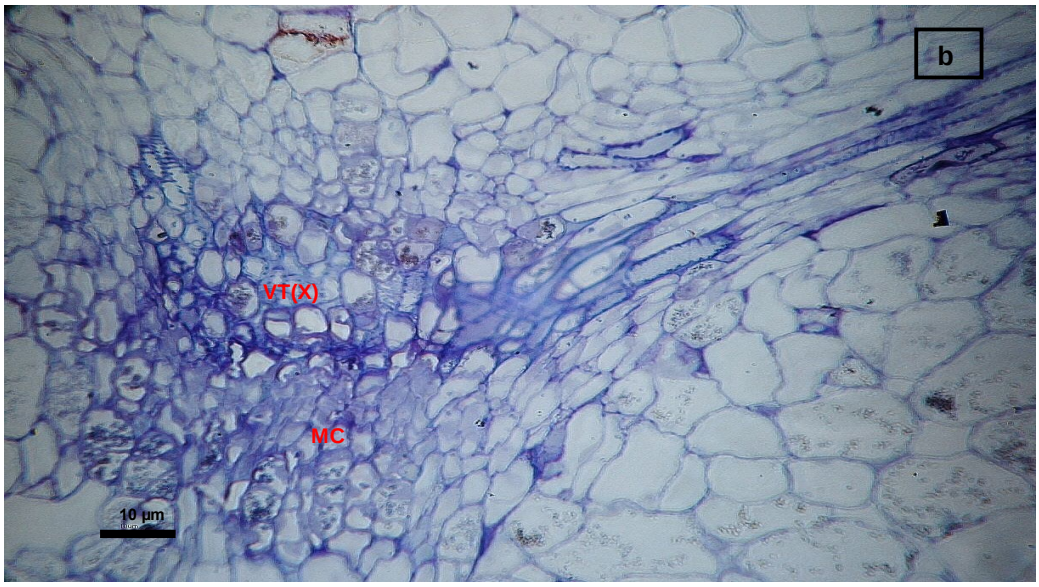


Figure 9: Different types of callus formed 29 days after initiation.9a,b,c,d - Crystalline callus(C). 9e,f - shiny/glassy (S/G) and brown/yellow/white callus.9e,f - Callus with brown/dark patches (P) on the surface. 9c,d,e – Nodular calli .





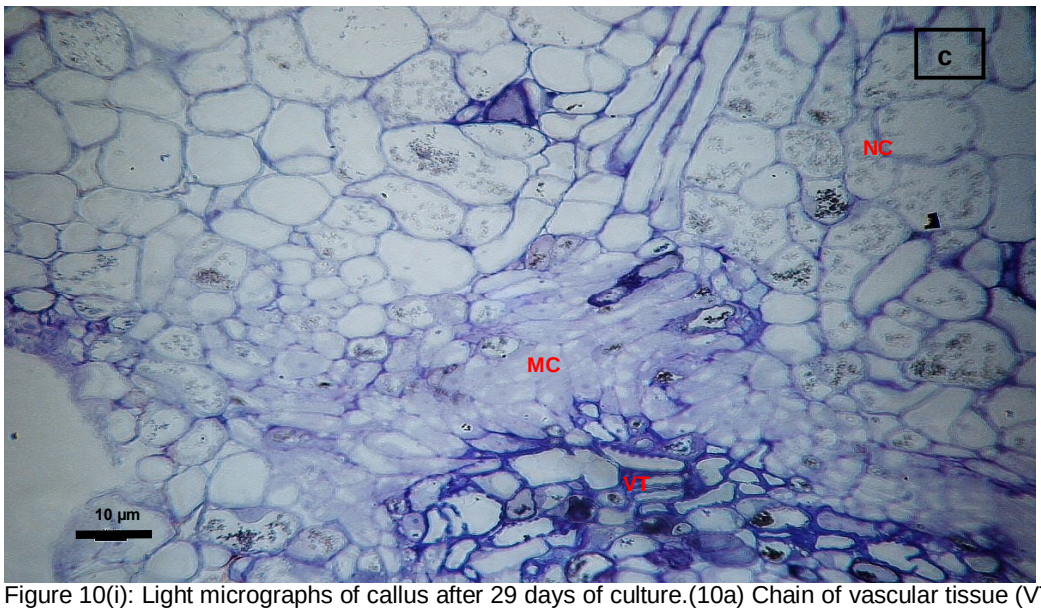
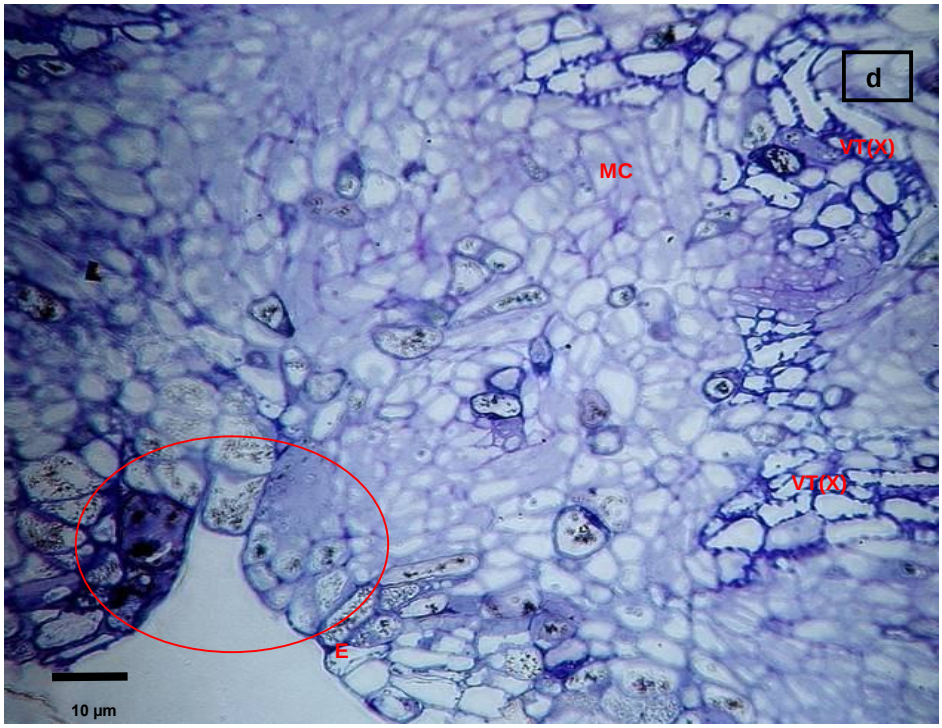


Figure 10(i): Light micrographs of callus after 29 days of culture.(10a) Chain of vascular tissue (VT) with xylem (X) connecting different parts of the callus.(10b,c) Vascular tissue (VT) connection to the morphogenic cells (MC),Nonmorphogenic cells (NC).



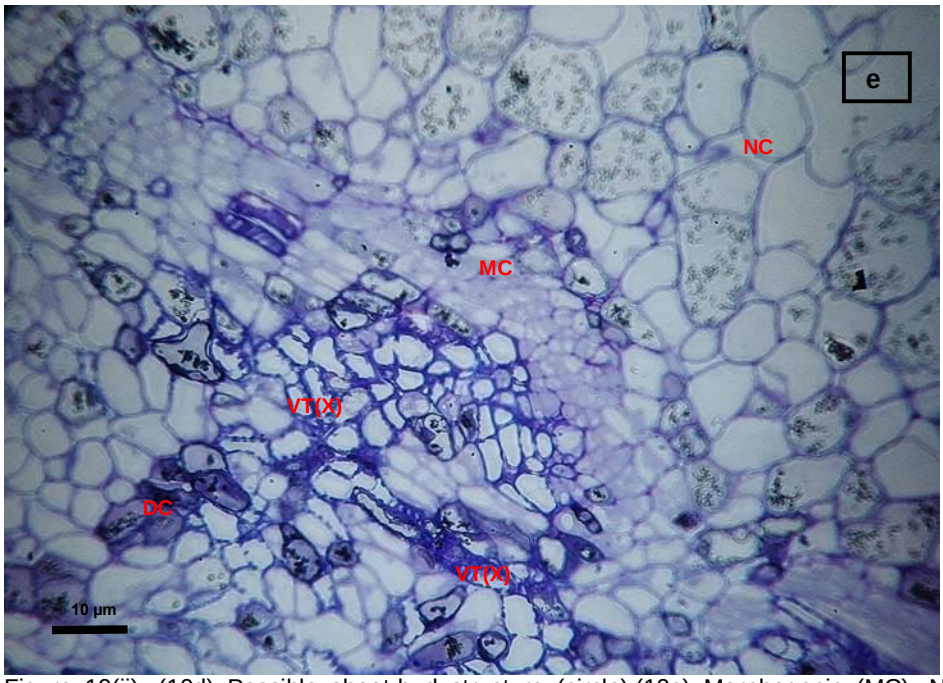


Figure 10(ii): (10d) Possible shoot-bud structure (circle).(10e) Morphogenic (MC), Nonmorphogenic (NC), Darkly stained cells, Vascular tissue (VT) with xylem (X).

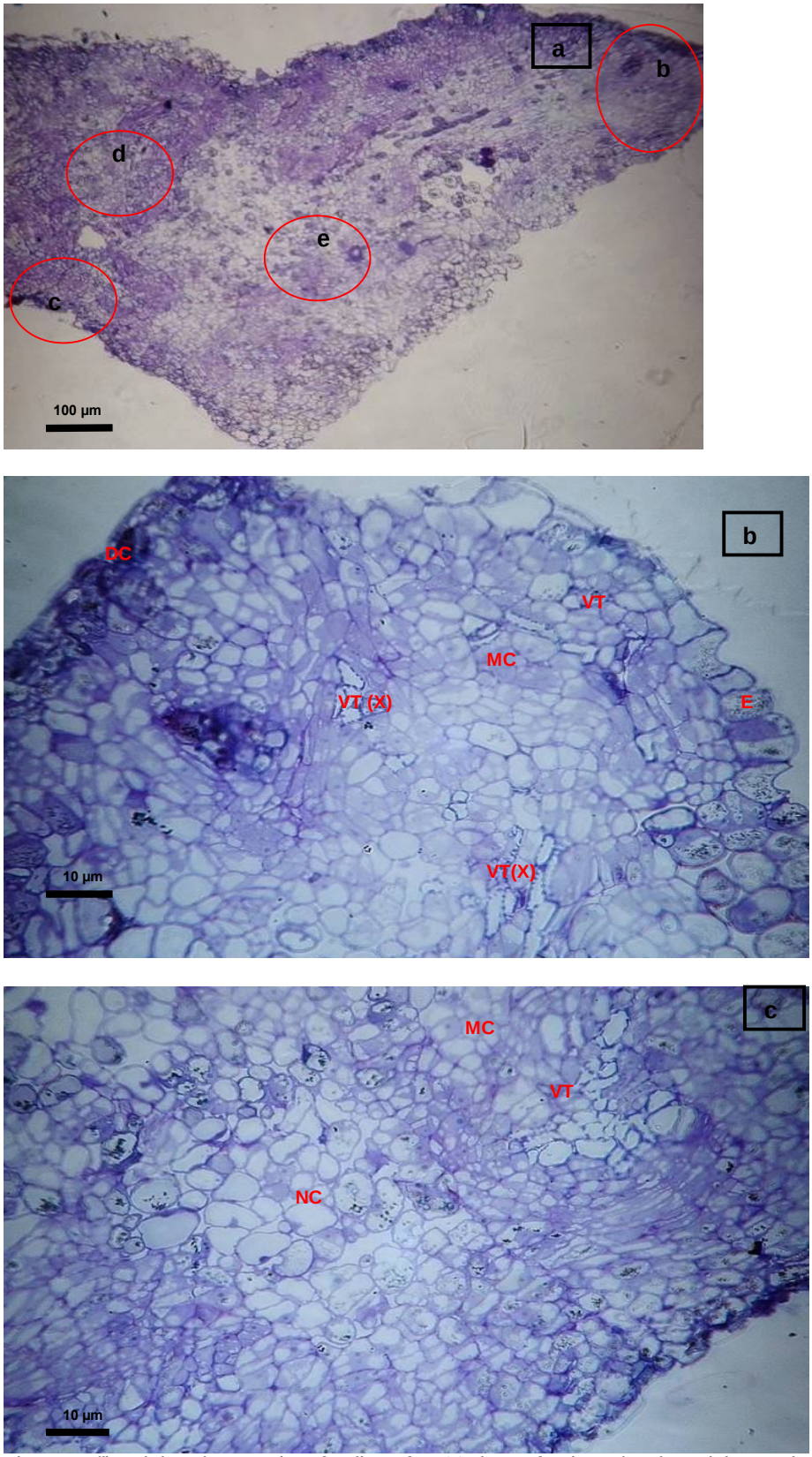
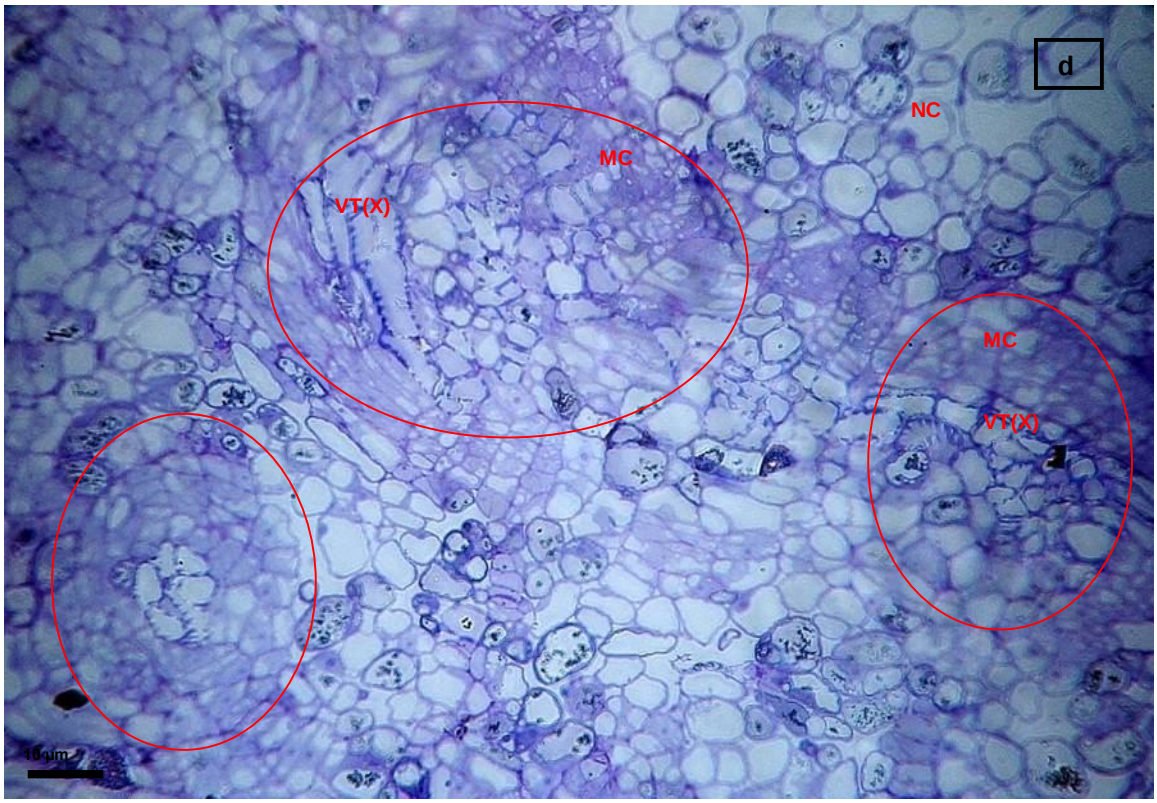


Figure 11(i): Light micrographs of callus after 29 days of culture.(11a) Nodular portion of callus.(11b,C) Nodular area on surface of calli with morphogenic cells (MC) associated with vascular tissue (VT) with xylem (X). Dark-stained cells (DC), Epidermis (E), Nonmorphogenic cells (NC).



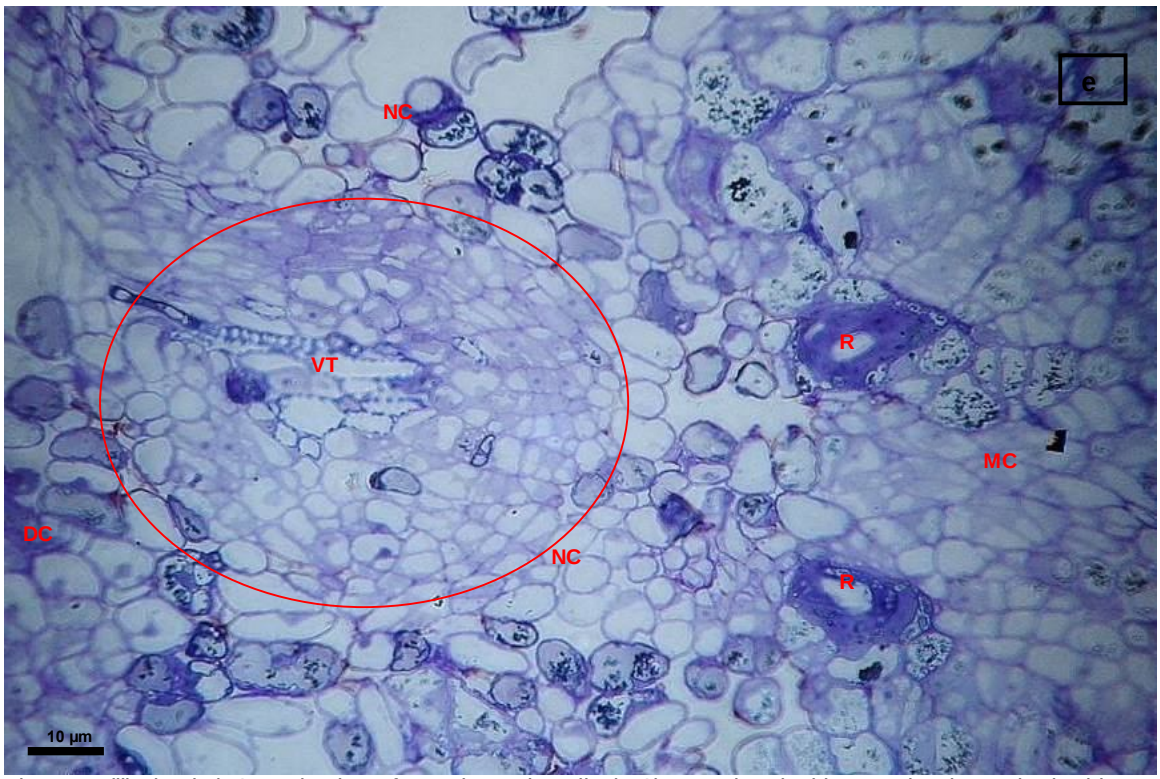


Figure 11(ii): (11d,e) Organization of morphogenic cells (MC) associated with vascular tissue (VT) with xylem (X) shown by circles. Nonomorphogegic cells (NC), Dark-stained cells (DC), Rounded packed cells (R) with central hole.

3.1.4 Development of callus after 36 days of growth and 6 days after first subculture.

At day 36, samples represented the material six days after the first subculture of the callus. Any adventitious shoot-buds that formed in the first 29 days were removed during the subculture to ensure that all the shoots were initiated from callus redifferentiation and not from any undestroyed axillary bud meristems. Callus explants (27%) developed shoot-buds. At this developmental stage, the callus became predominantly nodular (Fig. 12). As in days 15, 22 and 29 following callus types were identified: crystalline callus, glassy/shiny brown and yellow/white callus and callus with dark patches. Similarly the callus had greenish areas and this could be the initiation of a developing organ (Fig 12).

Morphogenic cells which had a close association with vascular tissue were also noted at this stage (Figs. 13a; 14a). These were identified as vascular nodules or clusters. The meristematic regions formed around the vascular tissue or a group of compact meristematic cells linked to vascular tissue (13a,b,c,d; 14a,b,c,d). The vascular nodules were induced towards the periphery of the callus and were responsible for the gross nodular appearance of the calli. Some of the calli were completely composed of tightly or packed meristematic cells (Fig. 15a,b,c) and these formed groups of meristematic nodular areas on the surface of the callus. As a general observation the calli typical of this stage were more developed and organized than the previous developmental stages.

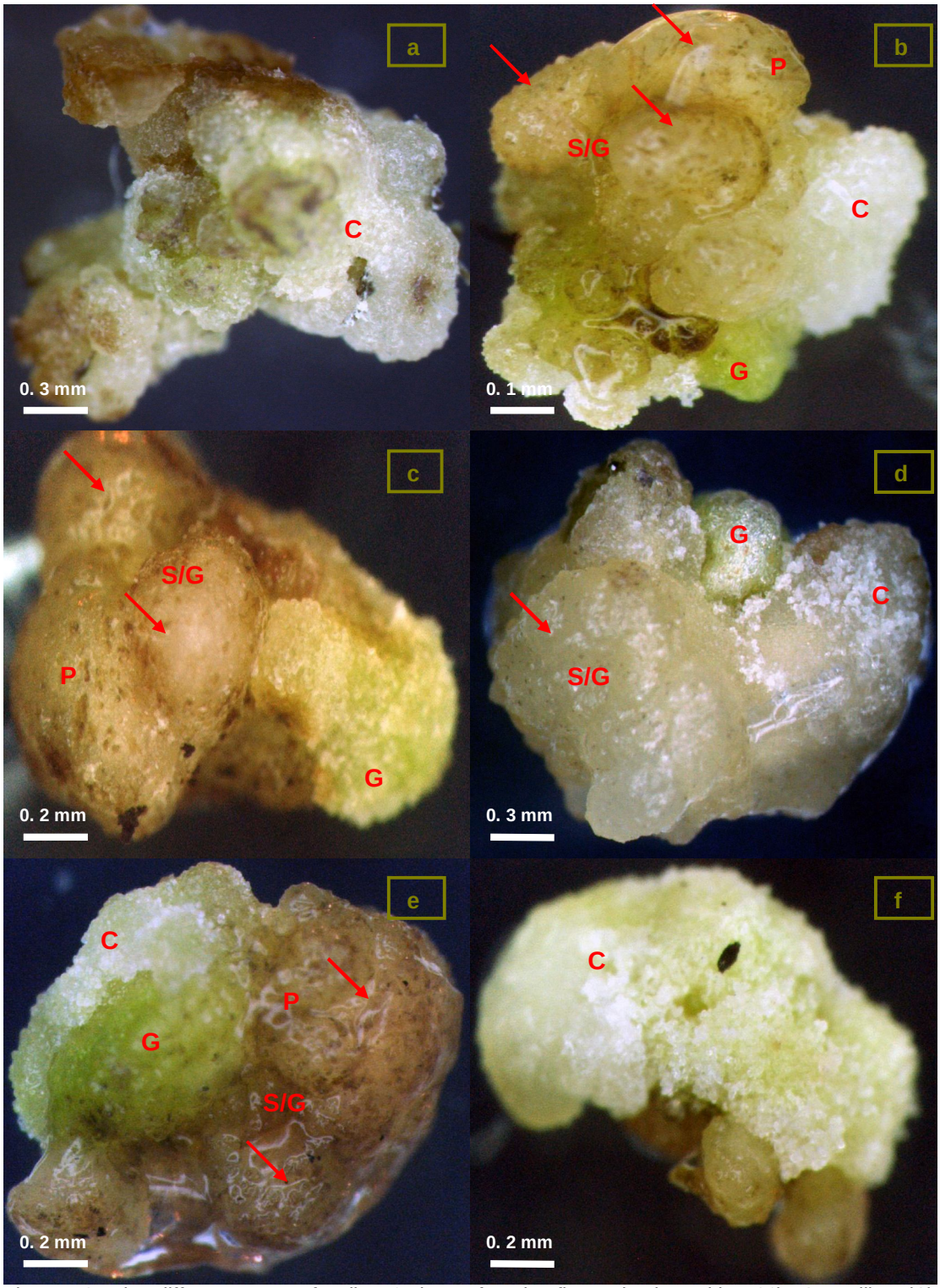


Figure 12: The different types of callus 6 days after the first subculture (day 36). Crystalline (C), shiny/glassy (S/G), Callus with patches (P), Nodular calli (represented by arrows), Greenish areas (G).

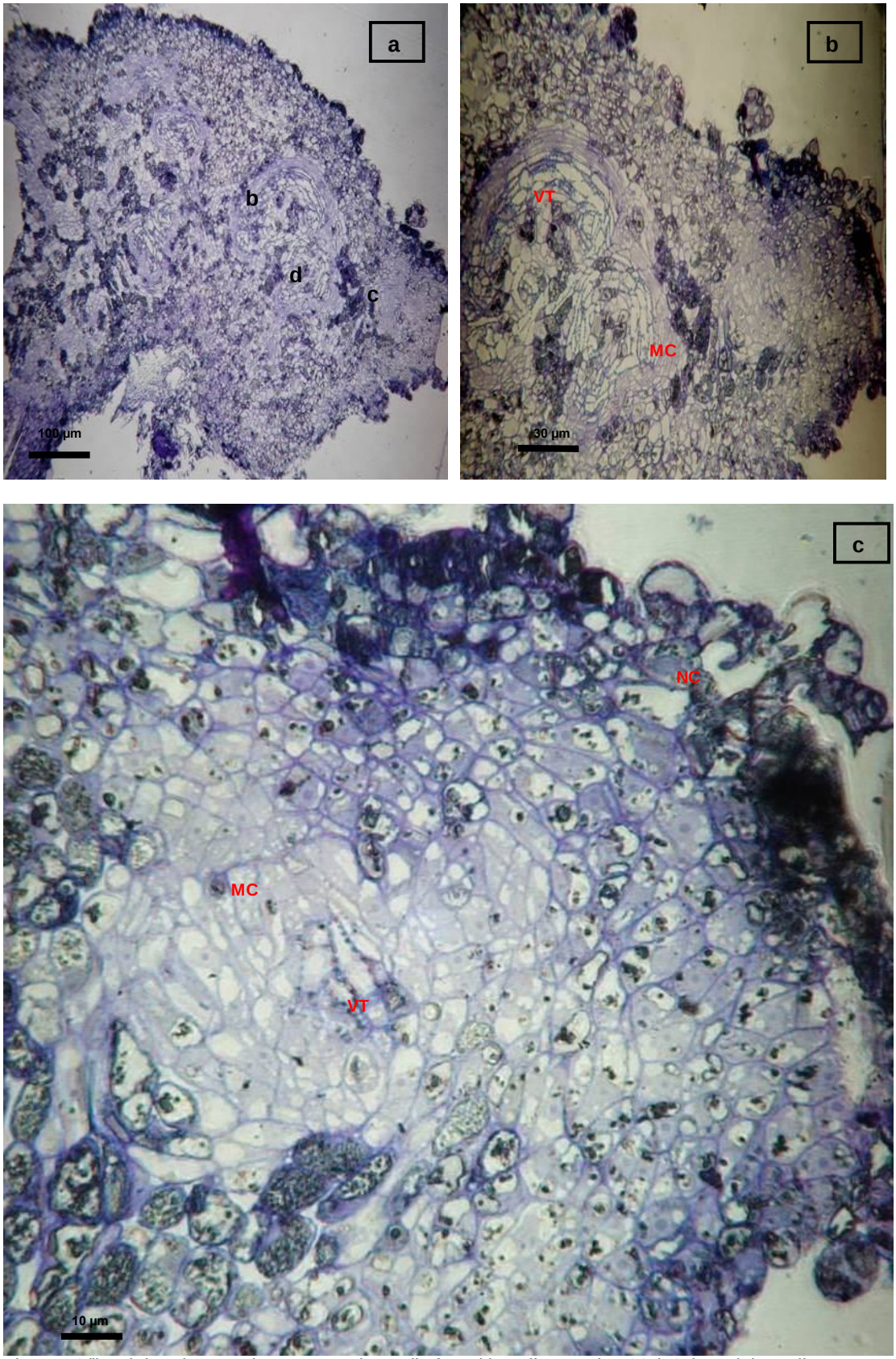


Figure 13(i): Light micrographs representing cells found in callus on day 36.(13a) Nodular callus. (13b,c) Morphogenic cells (MC) associated with vascular tissue (VT). Nonmorphogenic cells (NC).

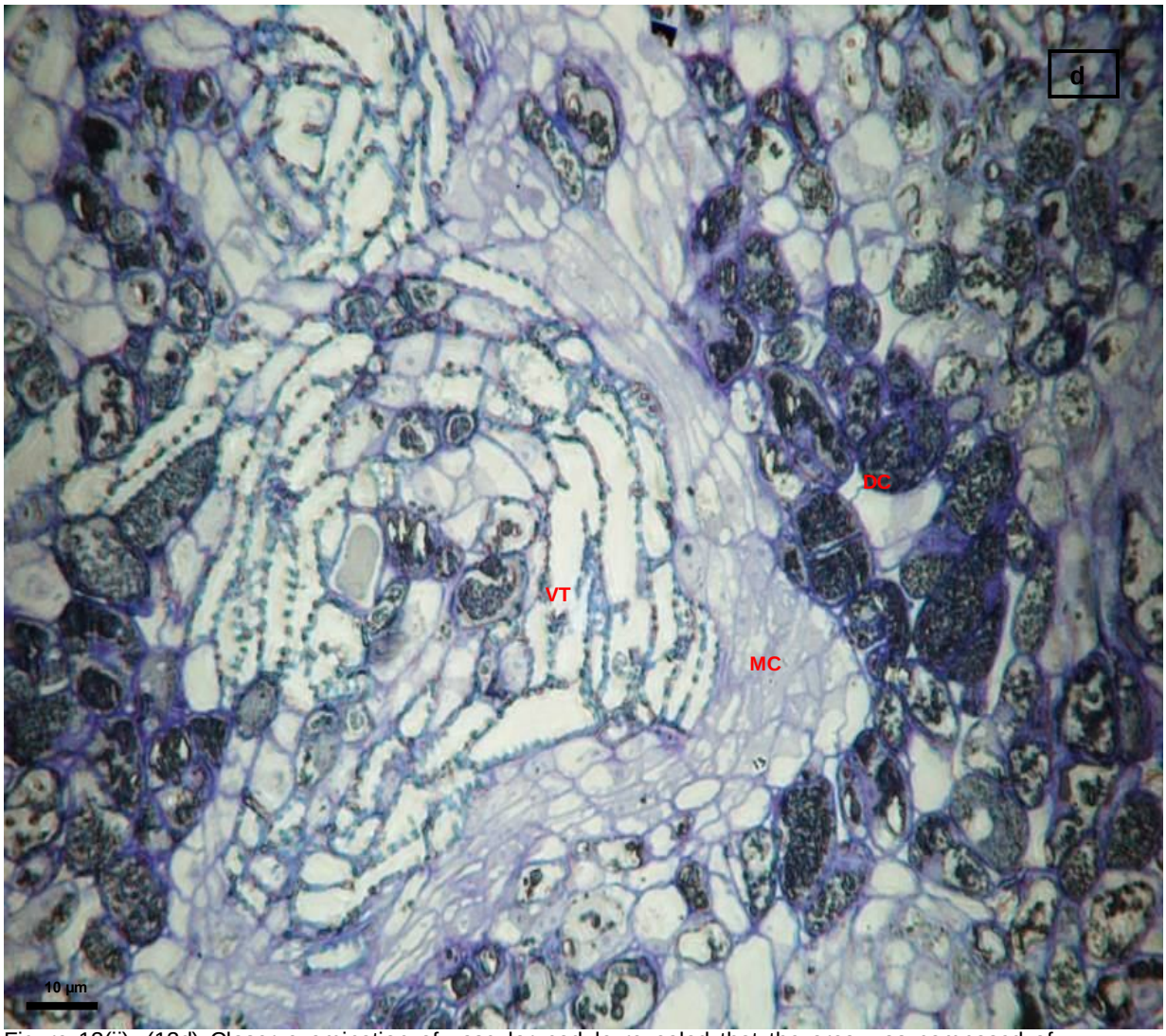


Figure 13(ii): (13d) Closer examination of vascular nodule revealed that the area was composed of morphogenic cells (MC) and vascular tissue (VT).Dark-stained cells (DC).

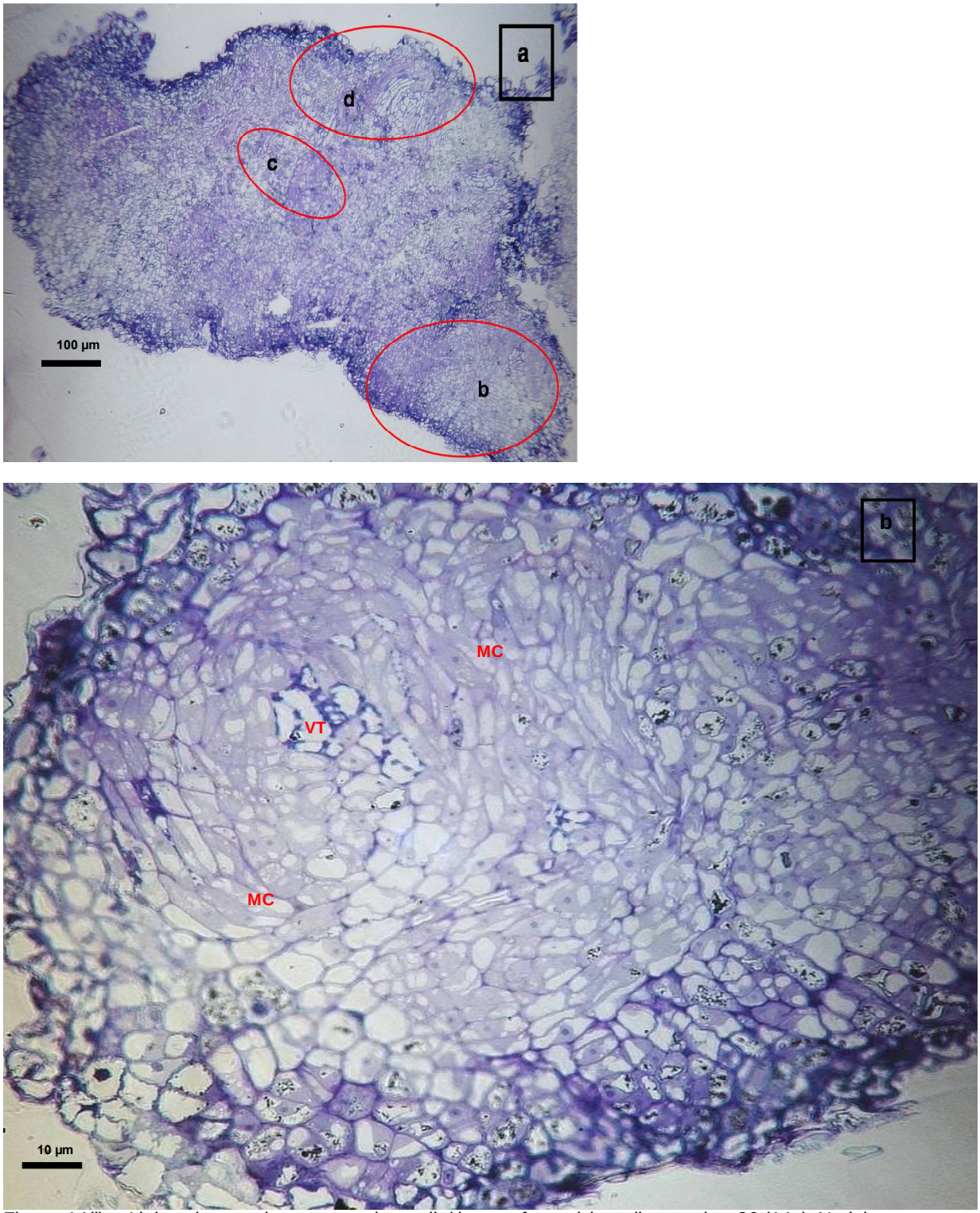


Figure 14(i): Light micrographs representing cells/tissues formed in callus at day 36.(14a) Nodular callus.(14b) Morphogenic cells (MC) associated with vascular tissue (VT).

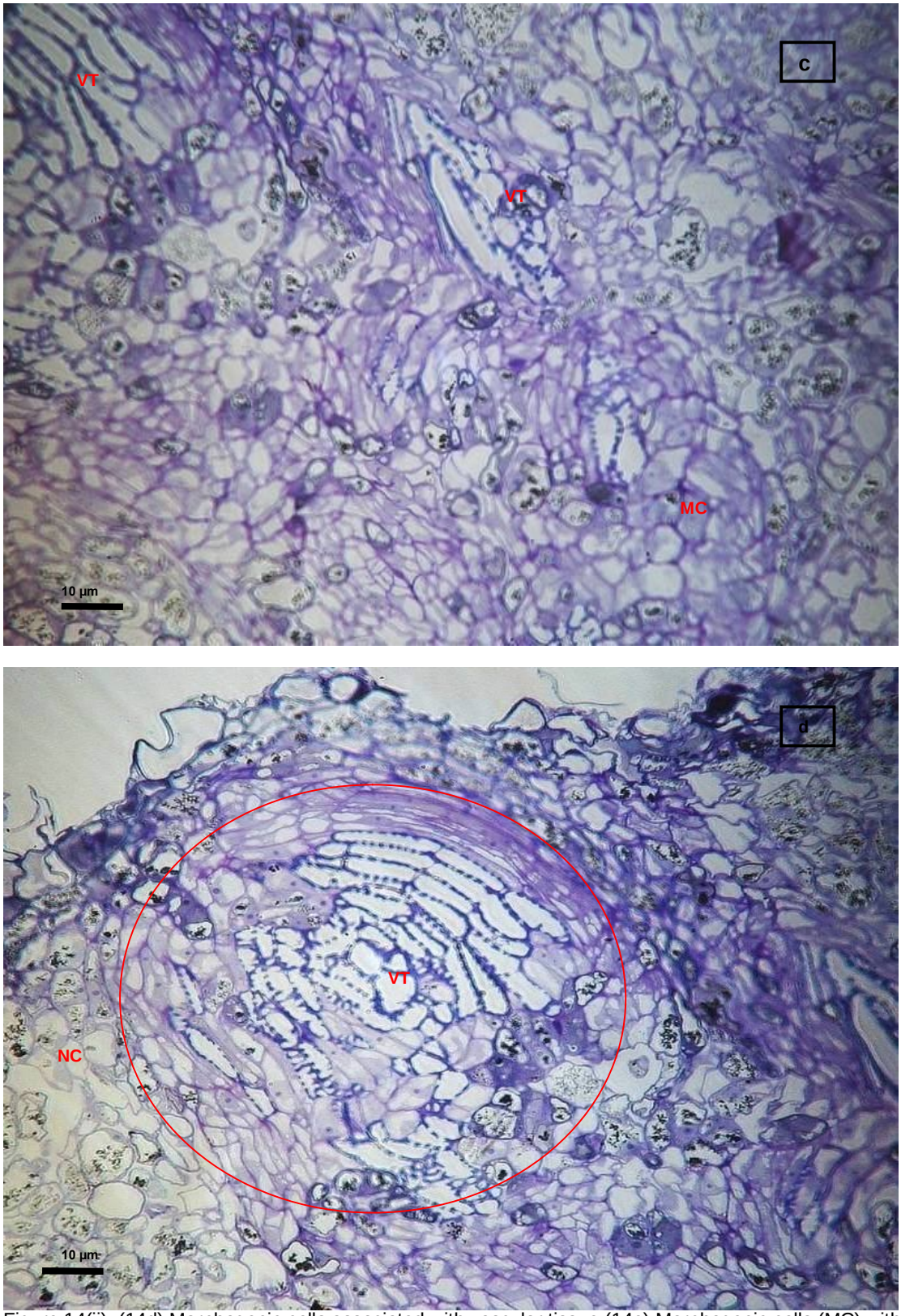


Figure 14(ii): (14d) Morphogenic cells associated with vascular tissue.(14e) Morphogenic cells (MC) with vascular tissue (VT) forming a vascular nodule (circles). Nonmorphogenic cells (NC).

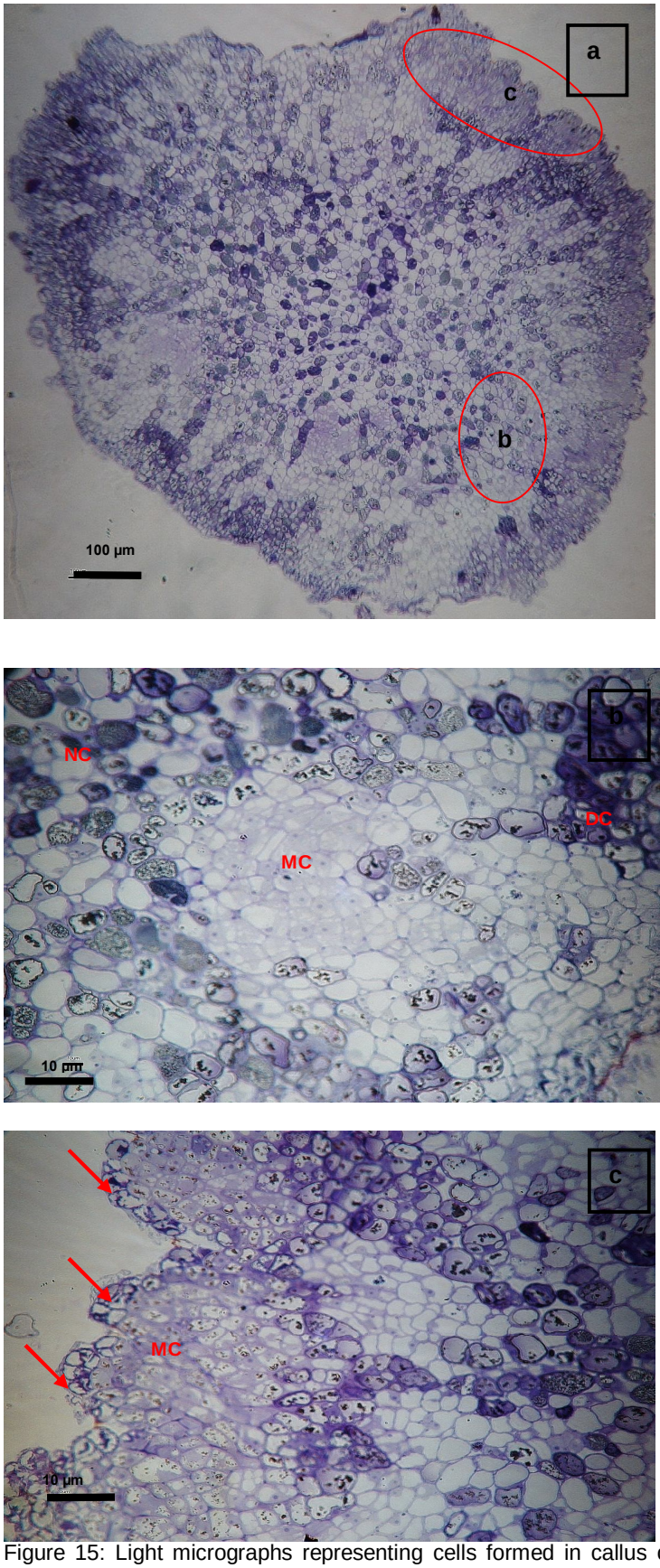


Figure 15: Light micrographs representing cells formed in callus on day 36.(15a) Section through a nodular meristematic callus.(15b) Morphogenic cells (MC), dark-stained cells (DC), nonmorphogenic cells (NC).(15d,e) Meristematic nodules on the surface of callus (arrows).

3.1.5 Developmental process of callus after 43 days of growth.

At this sampling time, there was an increase from 27 to 28% of callus that regenerated shoot-bus (Figs. 16e,f). Nodular structures (16a,b,c,d) protruded from the surface of the calli with some calli having green areas (Fig16d). The shoot-bud like structures appeared to have been formed from the callus that had green areas (Fig. 16d). The greening of the callus could well be associated with organogenesis; since these green regions were meristematic and the associated cells contained visible plastids these were assumed to indicate development. Apart from crystalline, brown, yellow or white calli, a red pigmented callus was also identified (Fig. 16e). The red callus was also capable of developing shoot-buds. It is possible that the red callus comprised cells that had plastids that had differentiated into chromoplasts.

Clusters of vascular tissue with meristematic or morphogenic cells collectively forming “vascular nodules” as described for the previous stage (Figs. 17a,b,c,d,e) were also identified. The nodular areas were scattered in callus tissue and had expanded in size, which could be attributed to the proliferation of cells in the vascular nodules and forming part of the shoot-buds initials. Callus that was further developed produced shoot-bud like structures that appeared on the surface of calli and had developing buds and leaf primordia (Figs 18a,b&c).

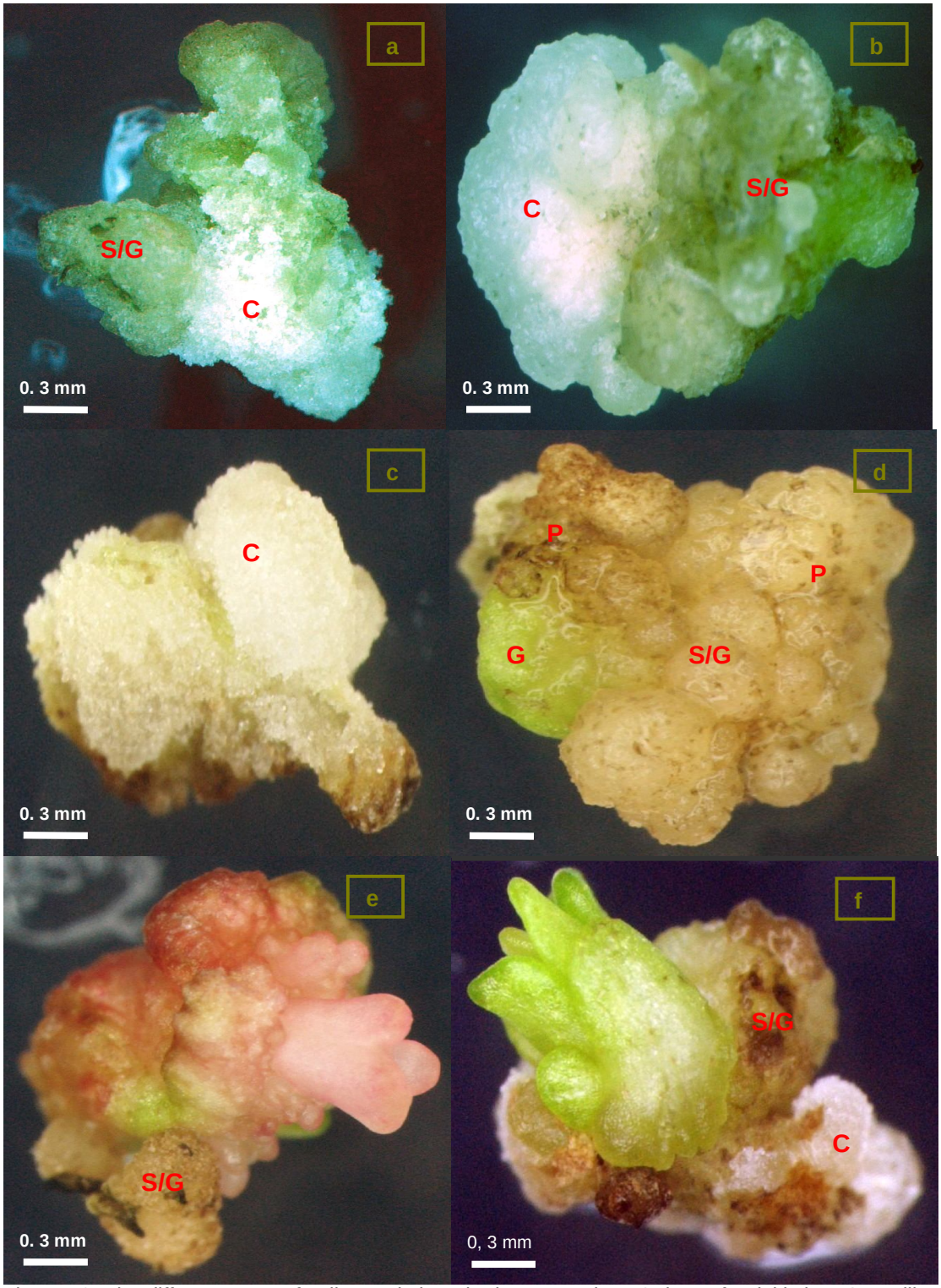


Figure 16: The different types of callus and shoot-bud regeneration 43 days after initiation. Crystalline (C), shiny/glassy (S/G), Callus with nodules (N), Callus with patches (P), Greenish areas (G).

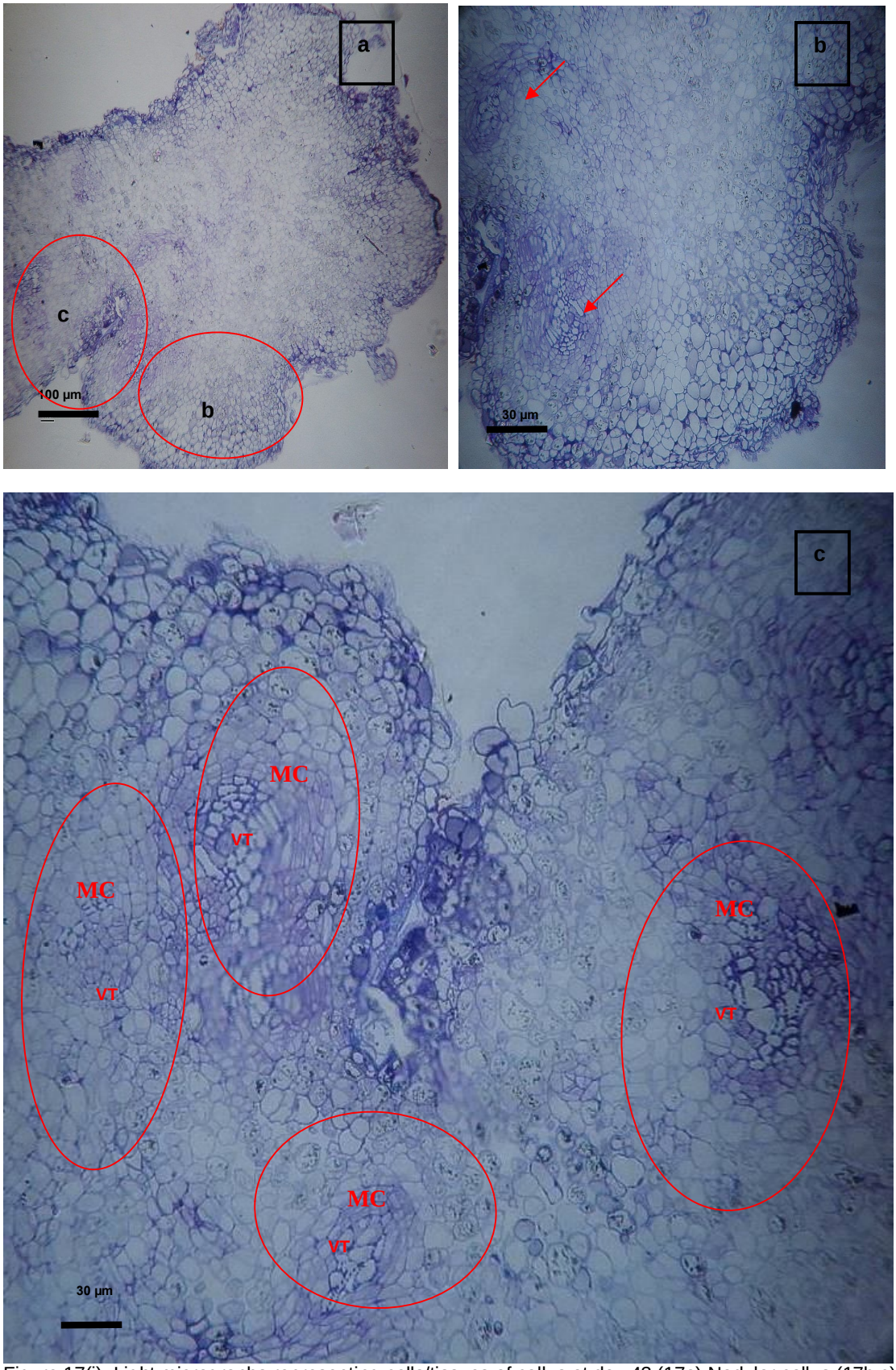
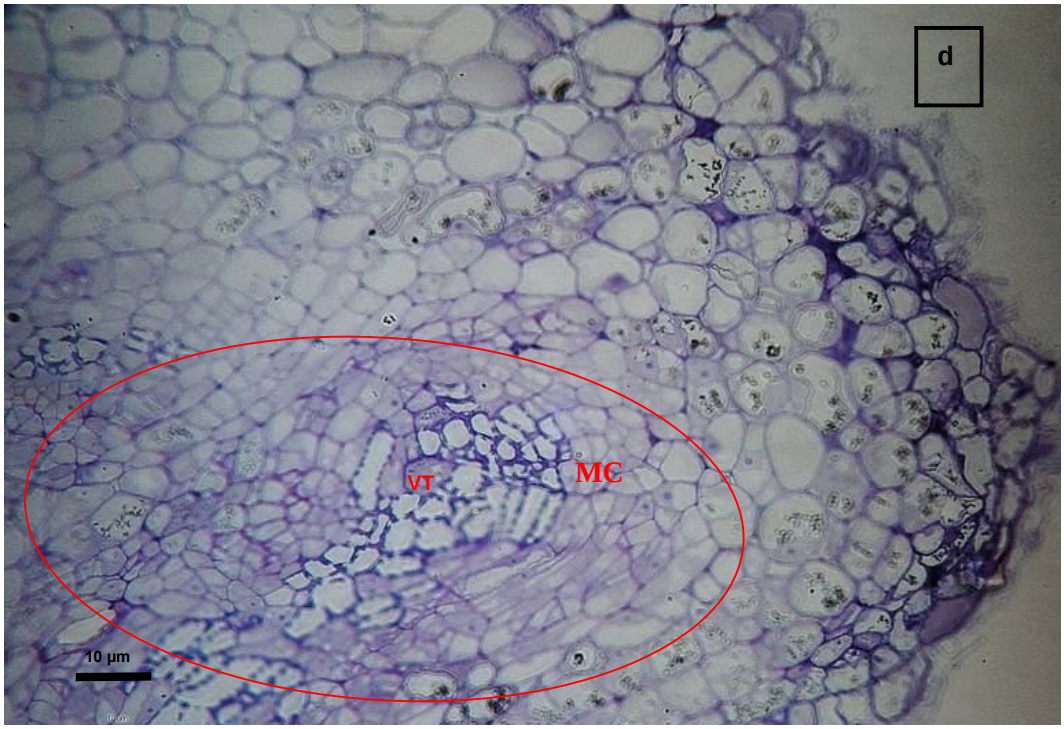


Figure 17(i): Light micrographs representing cells/tissues of callus at day 43.(17a) Nodular callus.(17b,c) Vascular tissue (VT) associated with morphogenic cells (MC) forming vascular nodules (arrows circles).



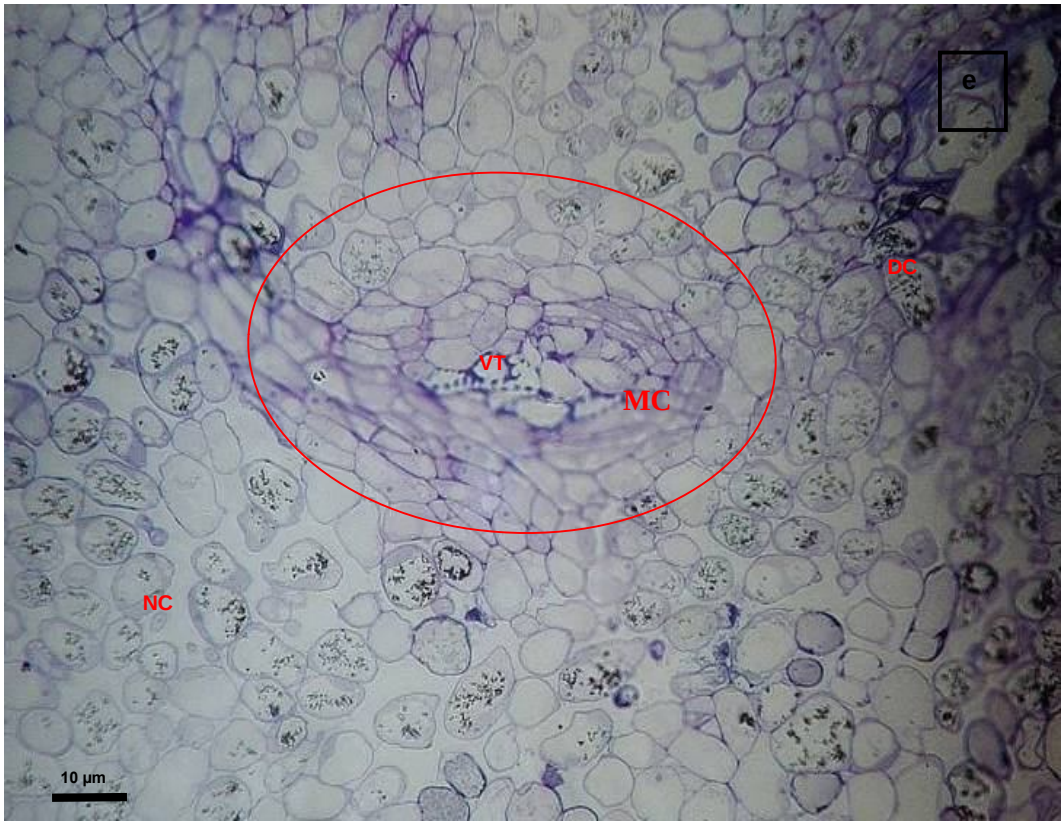
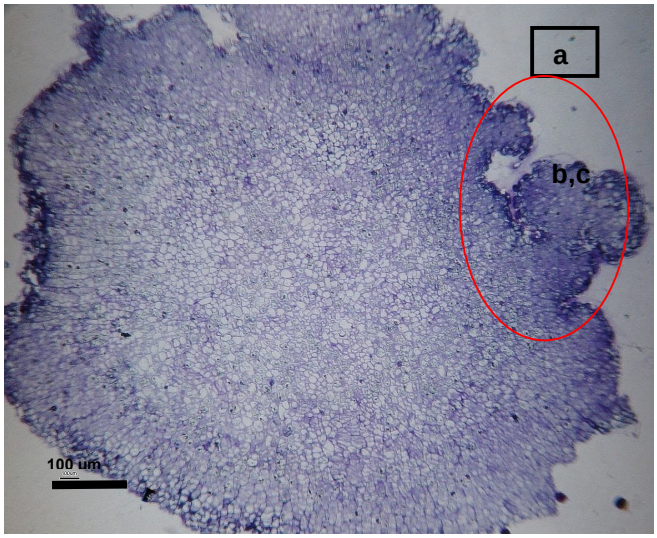
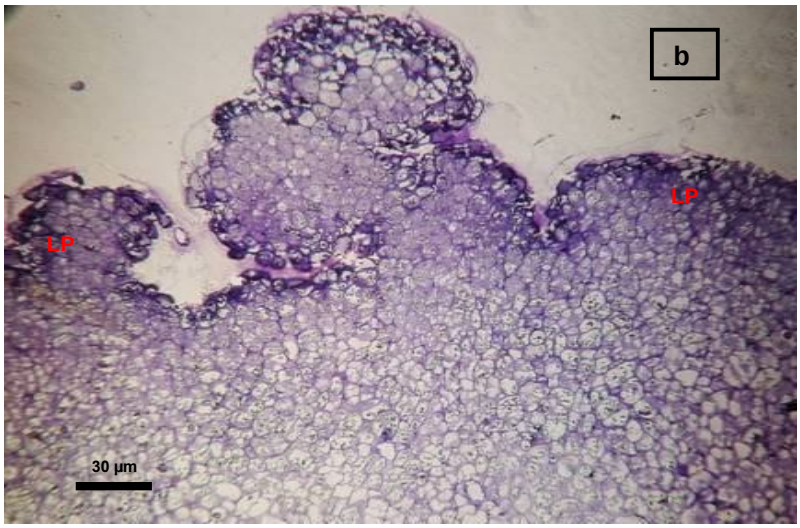


Figure 17(ii): (17d,e) Closer examination of vascular nodules showed that they were composed of morphogenic cells (MC) and vascular tissue (VT) (circles). Nonmorphogenic cells (NC), Dark-stained cells (DC).





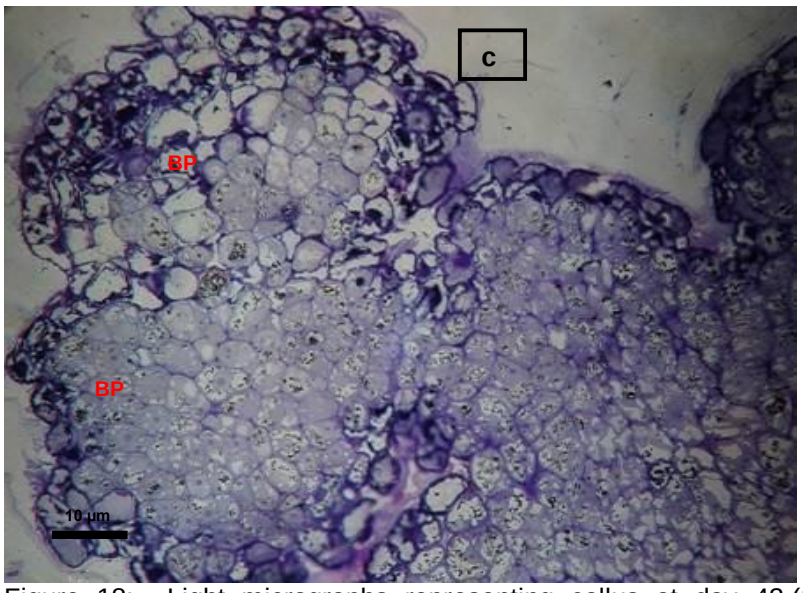


Figure 18: Light micrographs representing callus at day 43.(18a) Nodular compacted callus.(18b,c) Shoot-bud like structure with developing bud-primordia (BP) and leaf primordial (LP). Dark-stained cells (DC).

3.1.6 The development of shoot-buds from callus between 50 and 57 days.

Between the seventh and eighth week of culture, only 39% of calli produced adventitious shoot-buds, with one to three shoots forming per piece of callus (Fig. 19). The shoots protruded from the nodular and glassy callus and the crystalline layer covered some of the regenerative callus (Figs. 19a,b,c,d,e,f).

Clusters of vascular nodules were still noticeable in the callus that had not formed shoots and the cells were undergoing differentiation (Fig. 20a,b,c). A large mass of nonmorphogenic cells did not form part of the clusters but were loosely arranged surrounding the vascular nodules (Fig. 20c). Histological analysis of callus with shoot-buds showed that the shoot-buds emerged next to vascular tissue and this indicated that a vascular connection was formed (Figs. 22a,b; 23a,b; 24a,b). The shoots that developed had normal apical domes that were enclosed by a pair of leaf primordia (Figs. 21; 22; 23 & 24). The first sub-epidermal outer layer of the apex was recognized as the tunica and the second and third inner layers as the corpus. The tunica and corpus consisted of densely stained meristematic cells. Globular-like structures also developed on the surface of the apical dome (23b).

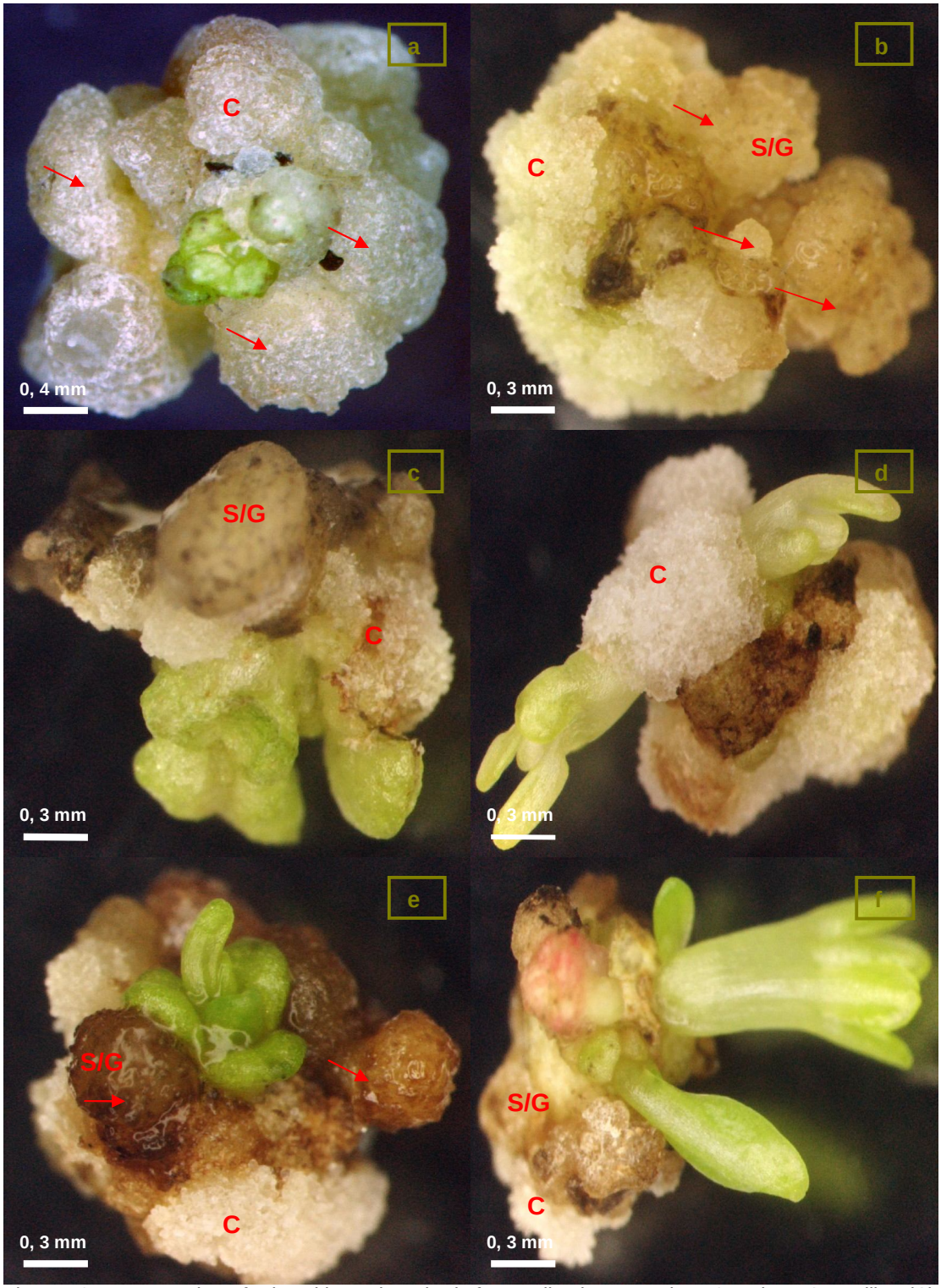


Figure 19: Regeneration of adventitious shoot-buds from callus between day 50 and 57. Crystalline (C), Shiny/glassy (S/G), Callus with nodules (N) represented by arrows.

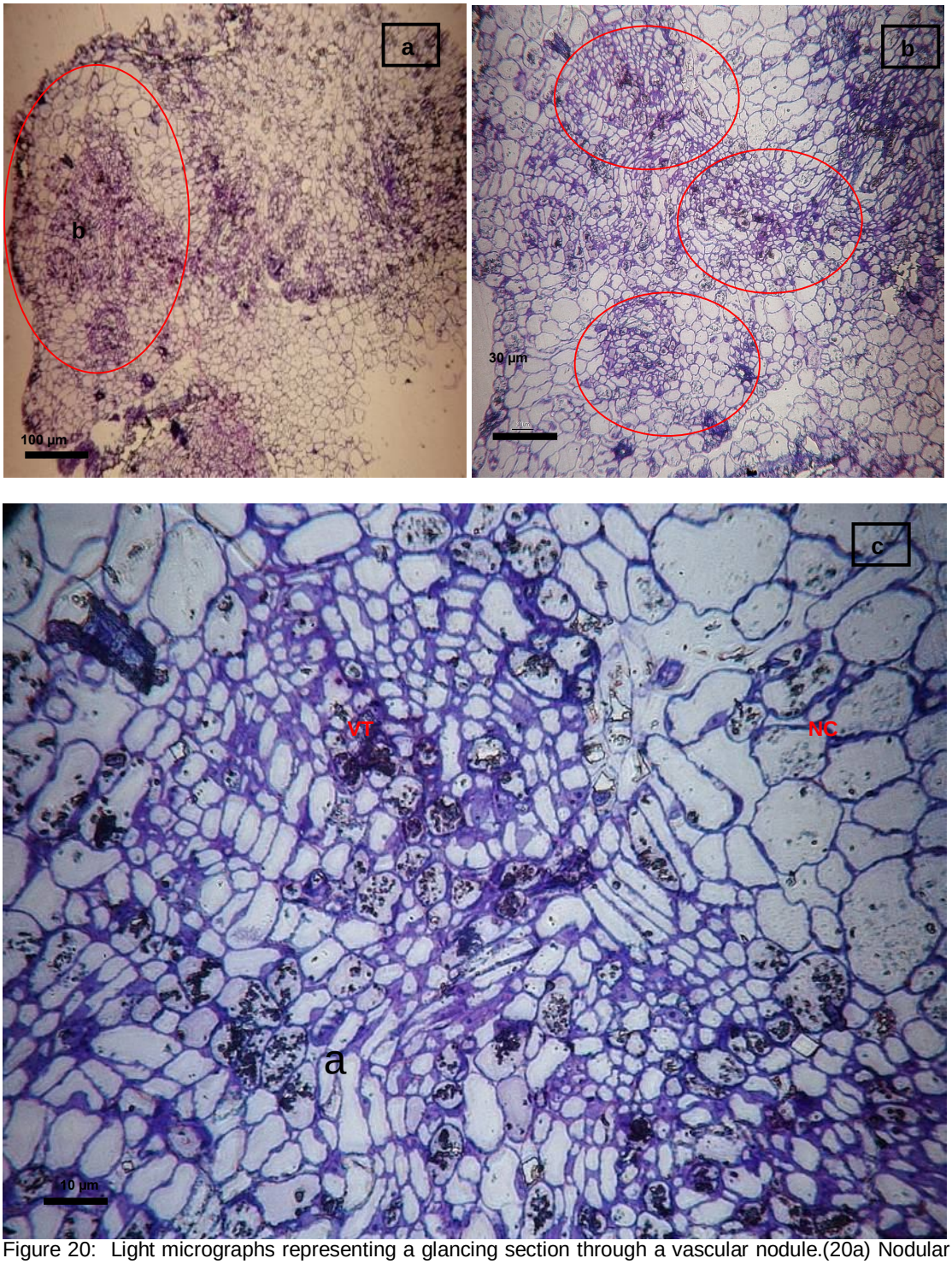
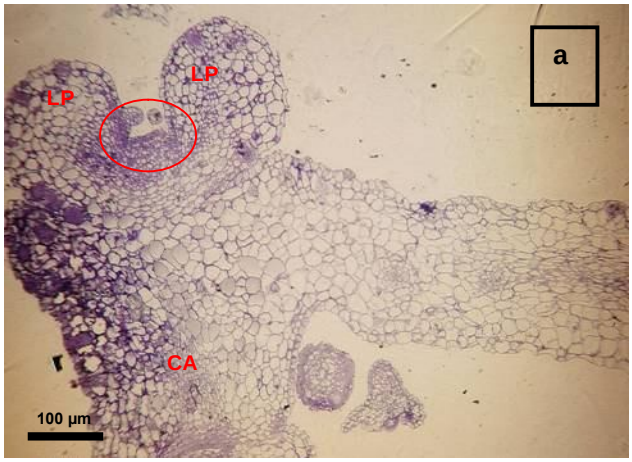


Figure 20: Light micrographs representing a glancing section through a vascular nodule.(20a) Nodular callus.(20b) Vascular nodules (circles).(20c) Vascular tissue (VT) and nonmorphogenic cells (NC).



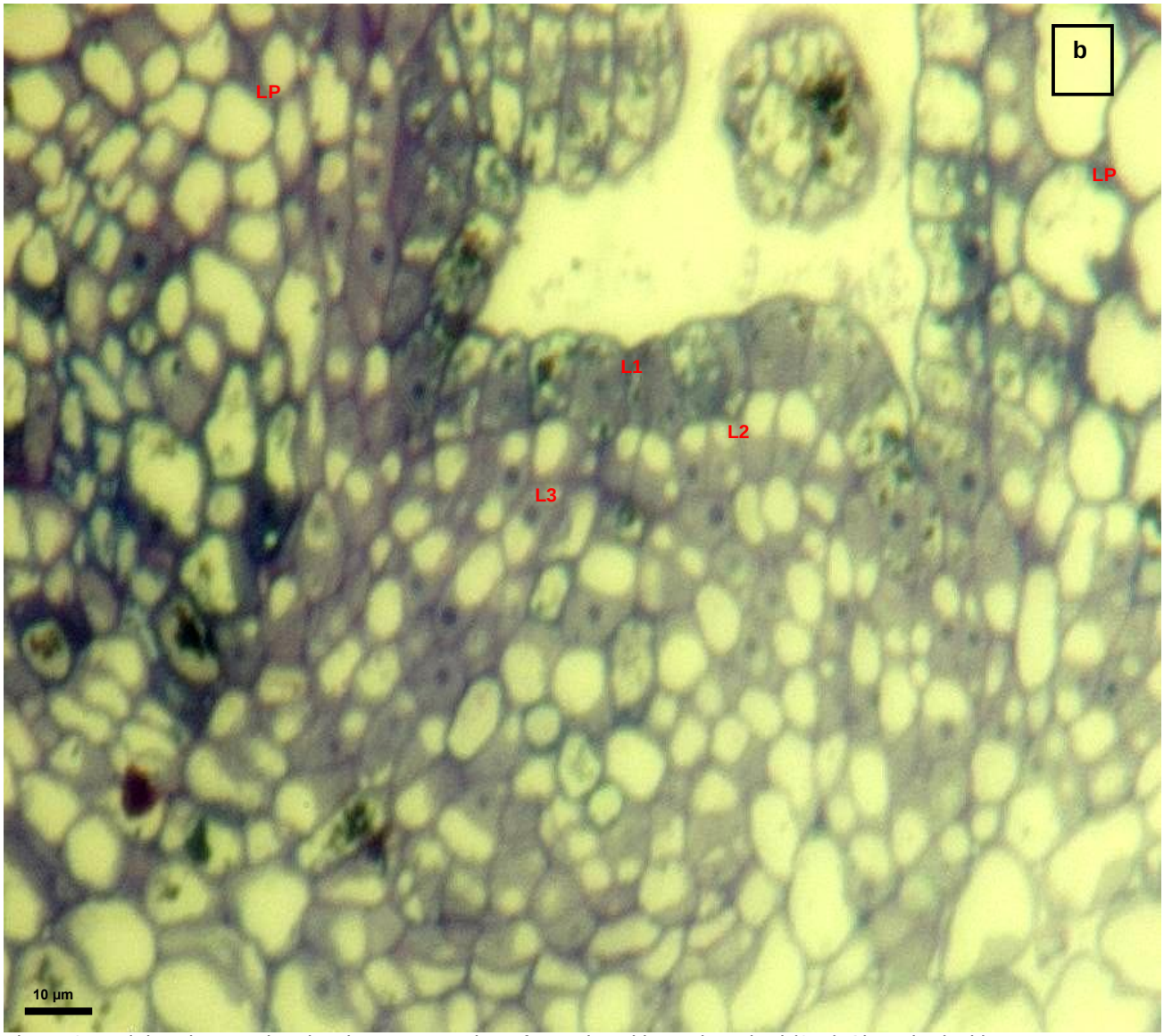
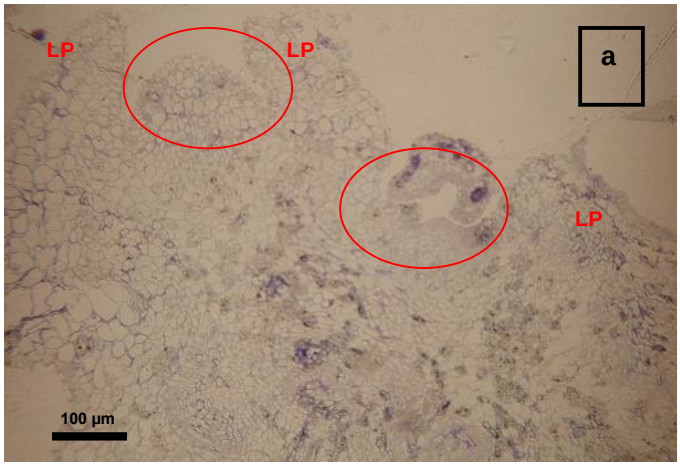


Figure 21: Light micrographs showing regeneration of an adventitious shoot-bud.(21a) Shoot-bud with two leaf primordia and apical dome (circled).(21b) Shoot apex with three meristematic layers (L1,L2,L3). L1-tunica, L2,L3- Corpus. Leaf primordium (LP), Callus (CA).



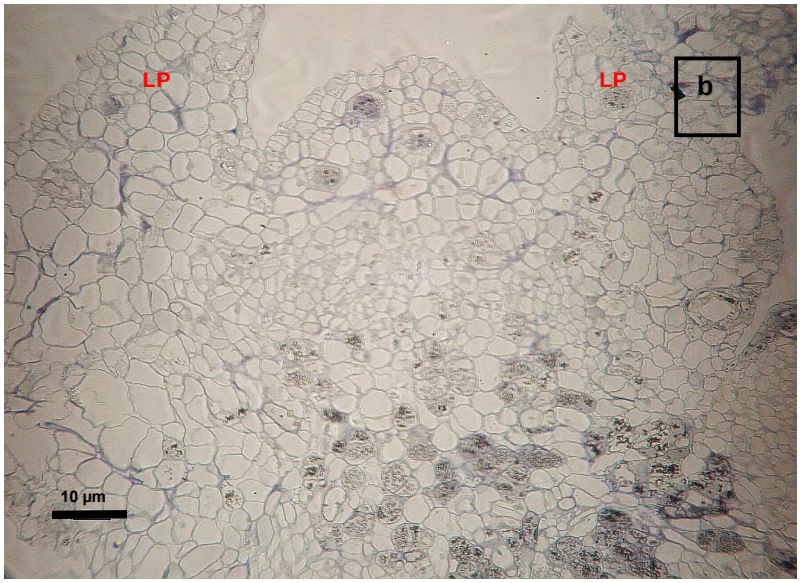




Figure 22: Formation of shoot-buds from callus (circles).(22a,b,c) Two shoot-buds with leaf primordial (LP). (22b) Shoot apex with the characteristic three meristem layers (L1, L2, L3) and vascular tissue (VT). L1- Tunica, L2,L3-Corpus.

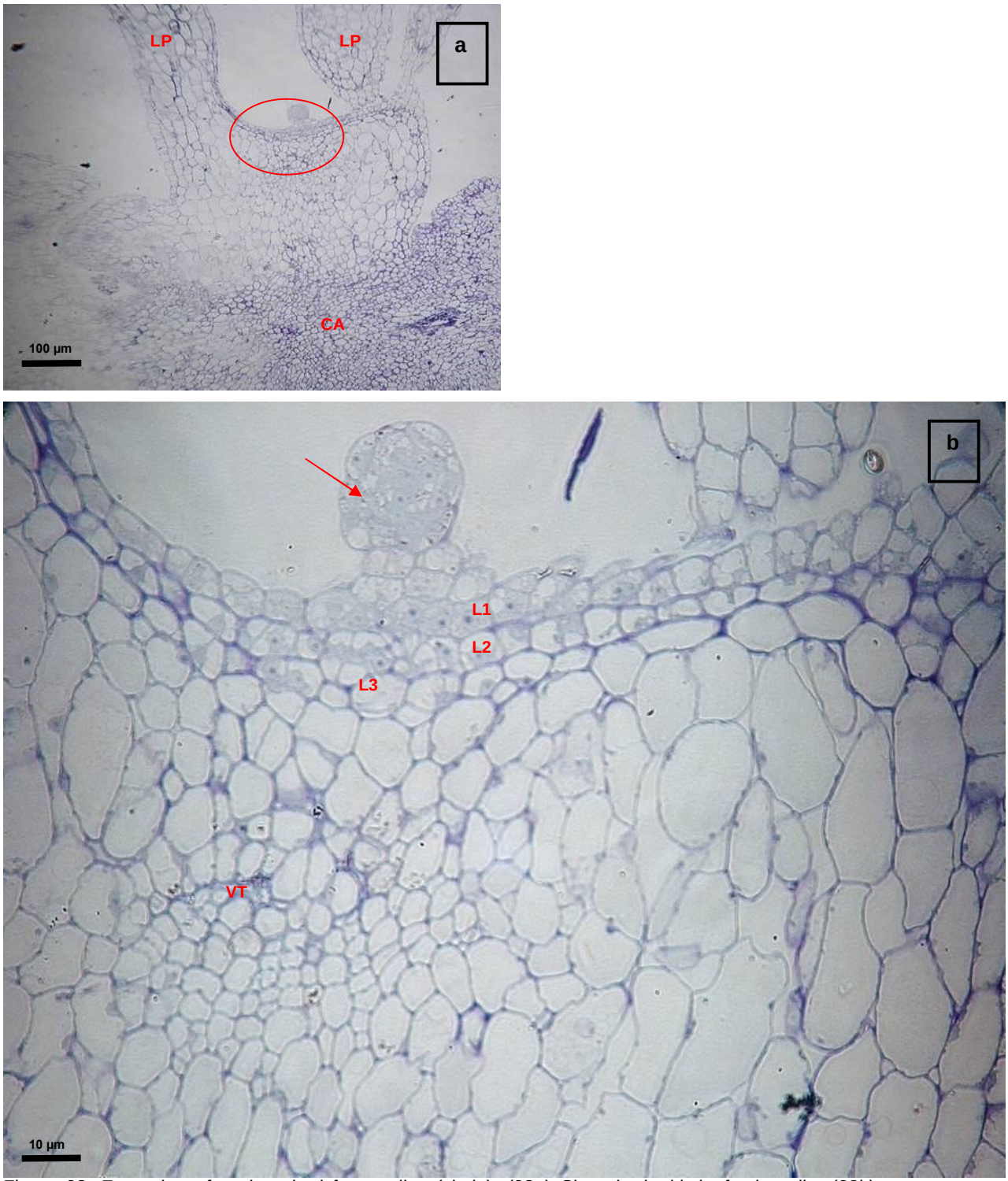
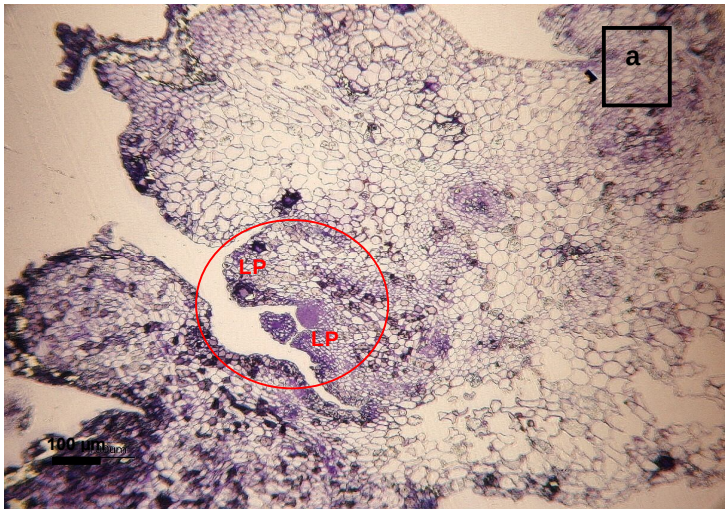


Figure 23: Formation of a shoot-bud from callus (circle). (23a) Shoot-bud with leaf primordia. (23b) Shoot apical region with three layers (L1,L2,L3) and globular structure (arrow). Vascular cambium was developed. L1-Tunica, L2,L3- Corpus. Leaf primordium (LP), Vascular tissue (VT), Callus (CA).



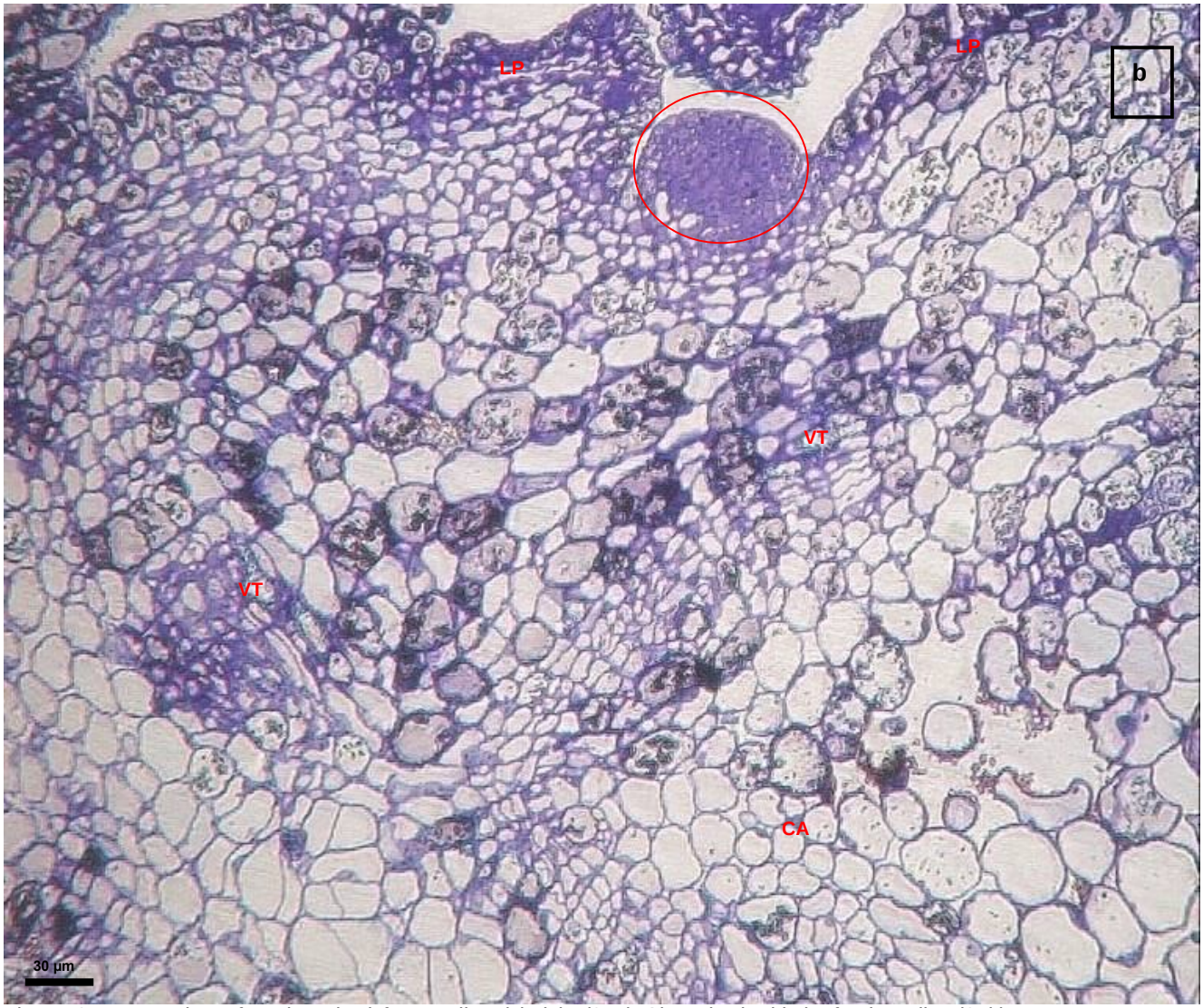


Figure 24: Formation of a shoot-bud from callus (circle). (24a) Shoot-bud with leaf primordia. (24b) Apical meristematic cells with vascular tissue directly linked to the developed shoot-bud. Leaf primordium(LP), Vascular Tissue (VT), CA (Callus).

3.1.7 Summary

Table 3: Summary of callus and shoot development

TIME(Days)	GROSS MORPHOLOGY	HISTOLOGICAL DESCRIPTION
15	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface.	Vascular tissue, morphogenic, nonmorphogenic and dark-stained cells.
22	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface, nodular callus (white, yellow and brown). Green areas.	Vascular tissue, dividing morphogenic cells, nonmorphogenic cells, meristematic cells that formed nodular structures and shoot-bud like structures, epidermal-like cells.
29	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface, nodular callus (white, yellow and brown). Green areas.	Vacular tissue strands and Vascular nodules, parenchymatous/meristematic morphogenic cells, nonmorphogenic cells, epidermal-like cells.
36	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface, nodular callus (white, yellow and brown). Green areas.	Vascular nodules with morphogenic cells and vascular tissue, nonmorphogenic cells, compacted meristematic cells, epidermal-like cells.
43	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface, nodular callus (white, yellow and brown), red callus. Shoot-buds. Green areas.	Initiation of shoot-buds. Vascular tissue, vascular nodules, compact parenchymatous/ meristematic cells.
50-57	Callus appeareance: crystalline, glassy/shiny compact (white, yellow and brown), compact callus (white, yellow and brown) with dark spots on the surface, nodular callus (white, yellow and brown), Red callus. Shoot-buds.	Shoot-buds, parenchymatous/meristematic cells, vascular nodules, nonmorphogenic cells, globular-like sructure, tunica and corpus layer.

3.2 Structure of the regenerated stems

Stems derived from callus that were between 1-10 mm in height and 1-4 mm in thickness were used to describe the histology of the *Eucalyptus* stem after three days on rooting medium. The stems comprised the distinctive layers of epidermis, cortex and parenchyma tissues (Figs. 25 & 26a,b), typical of a dicotyledonous plant. The vascular cylinder in the centre showed traces of primary xylem and phloem in juxtaposition to the parenchyma tissues (Figs. 25 & 26a,b). Callus had formed at the cut ends and along the sides of the stem (Fig. 26c). It appeared that cells at the cut surface had undergone division and formed the basal callus (Fig. 26c). Xylem was associated with this callus (Fig 26c). In some instances the early stages of root primordium formation were observed at the base of the stems (Fig 26c). These meristematic regions appeared to have developed from the vascular cambium as had some callus (Fig 26b).

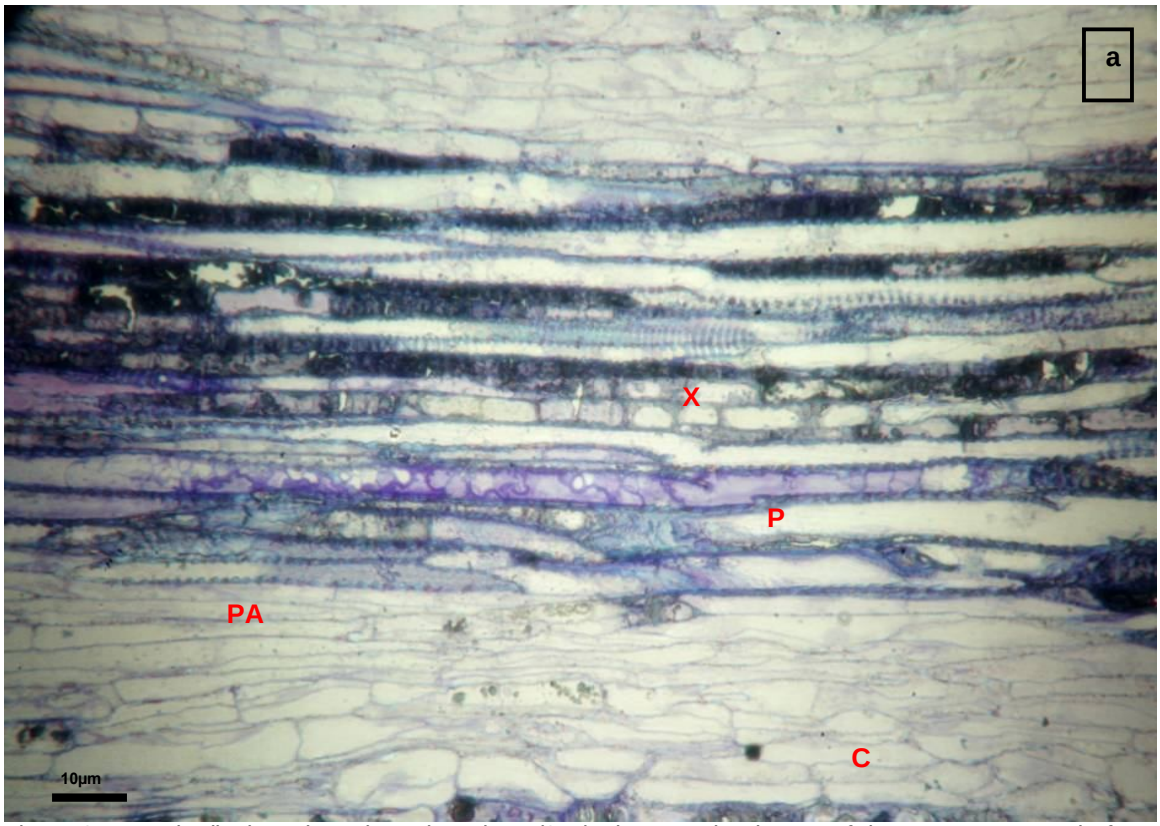


Figure 25: Longitudinal sections through a shoot.(25a) The vascular tissues of the stem composed of xylem (X) and phloem (P) situated between layers of parenchyma tissue (PA) and cortex (C).

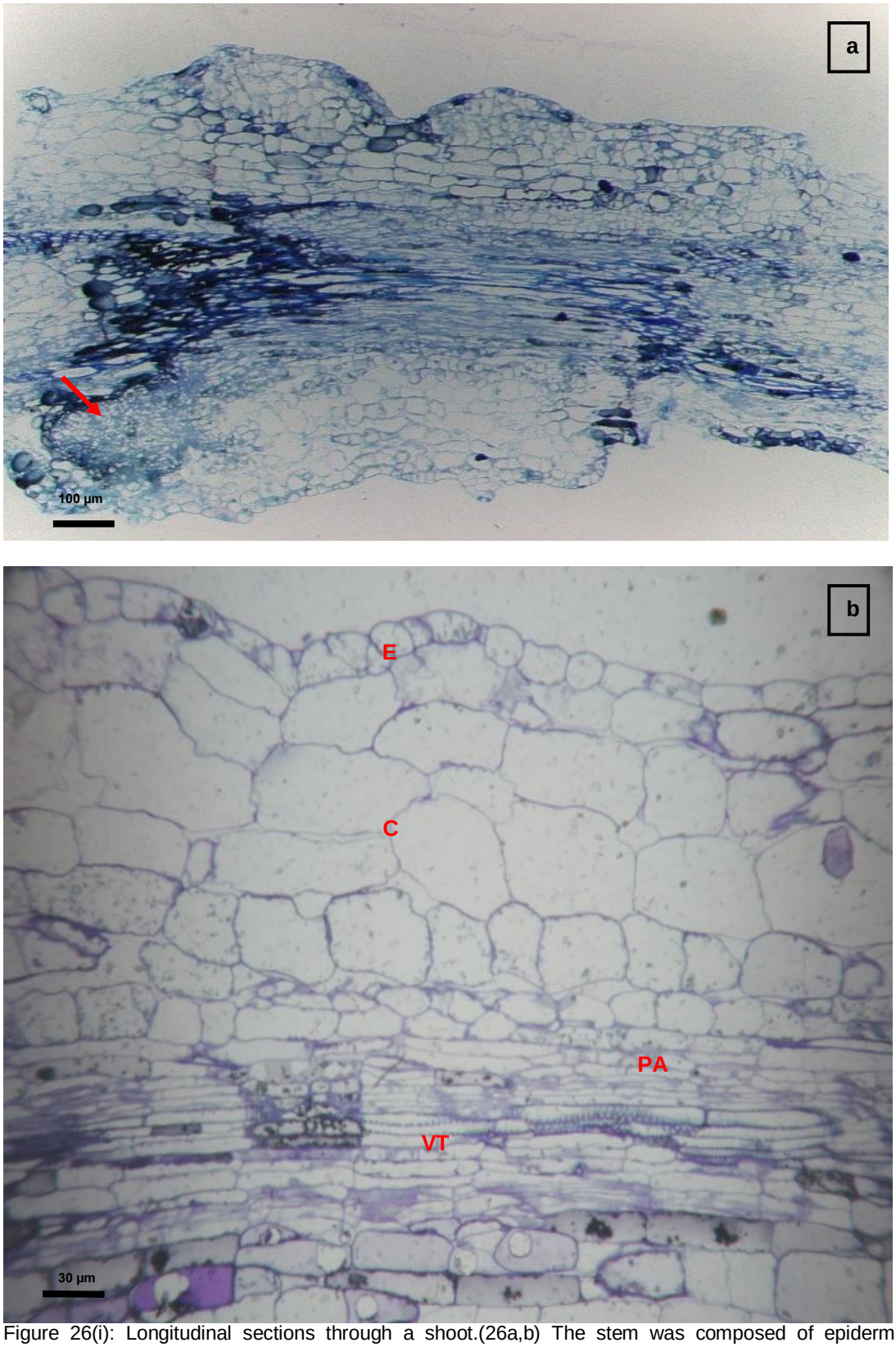


Figure 26(i): Longitudinal sections through a shoot.(26a,b) The stem was composed of epidermal layers(E), cortex (C), layers of parenchyma cells (PA) with vascular tissue (VT).

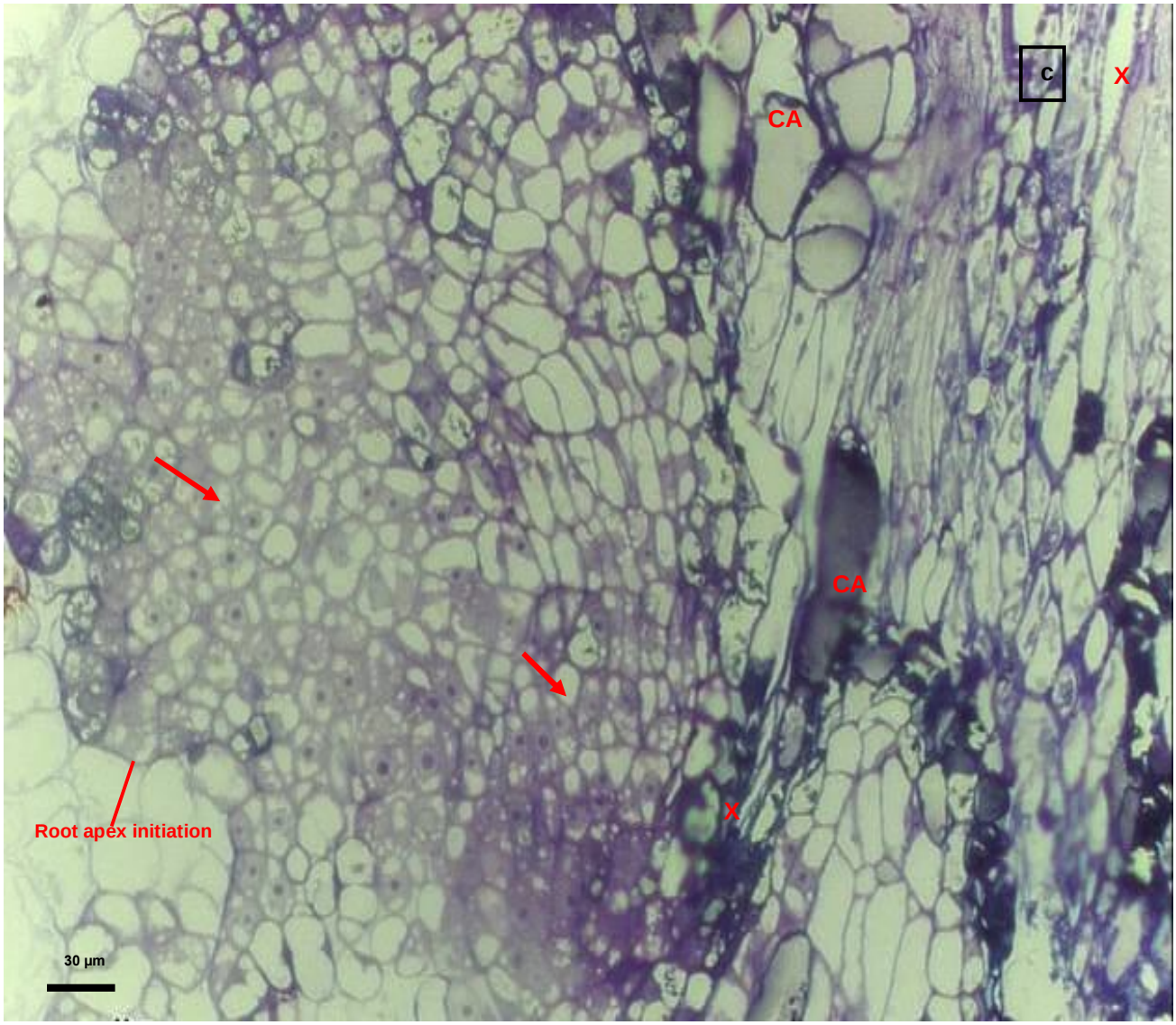


Figure 26(ii): (26C) Mitotic division (represented by arrows) associated with callus cells (CA) and vascular tissue (VT) (represented by arrows). Xylem (X).

3.3 Rooting

3.3.1 Morphology and histology of rooted shoots

After three days, the *in vitro* shoots (plated on hormone free medium) had developed roots of between 1-5mm in length, 1-2 roots per shoot. The total percentage of shoots that rooted was 74% after four weeks of culture (Fig. 27). All the shoots formed basal callus and this began forming within two days of culture in hormone-free medium (Figs. 28-32).

Developed roots had a root cap and a root apical meristem region characterized by layers of cells which contained central nuclei (Fig. 31b). The root cap cells were larger and wider when compared with the meristematic area. The cortex adjoining the vascular cylinder of the root comprised of large parenchymatous cells (Figs.30c;32e). The epidermal layer surrounded the root; in some areas appeared ruptured due to further development of roots (Figs. 29b).

After 24 hours on rooting medium, root regeneration followed two different morphogenic pathways, those that were formed either from callus re-differentiation at the base of the stem or from cambium and cortical parenchyma differentiation (Figs. 28a-c; 29a-e; 30a-f; 31a-b; 32a-f). The roots indicated an asynchronous development i.e. roots development at more than one stage of differentiation occurred in the same shoot (Fig. 29b,d,e). Lateral roots also emerged from developed adventitious roots (Fig. 32,c,d).

3.3.2 Structure of adventitious roots formed from callus differentiation but which lacked vascular connection to shoots

Half of the shoots sectioned showed no connection between the vascular system of the nascent root and that of the shoot (Figs. 28a,b,c; 29a,b,c). It is important to note that each sample was sectioned completely across its width. Within 3 days of culture the end of the stem darkened and formed callus at the base. The cells of the callus appeared large when compared with parenchyma tissue (Figs. 28c,d; 30d; 32e,f). Densely stained cells associated

with large irregular callus cells and vascular tissues were observed at the base of the shoots and were presumed a product of active cell division (Figs. 32e, f). These regions were meristematic with the cells showing prominent nuclei, and were presumed to be involved with the initiation of root primordia from callus re-differentiation. The light stained cells of the root primordia were presumed to be precursors of future cortical or vascular tissue and the developing root apex (Figs. 32e, f).

3.3.3 Structure of roots induced from cortical parenchyma and vascular cambium with a shoot/root vascular connection

Each sample was sectioned completely through and only 50% of the material and longitudinal observation showed that the vascular tissues of the shoot were continuous with the vascular cambium and parenchyma cells of the nascent roots (Figs. 30e,f,g; 32b). The areas of connection between the shoots and roots showed the presence of xylem and phloem tissues associated with parenchyma cells. A group of root initials observed were located nearest to the vascular tissue of the parental root (Fig. 29 d, e). The root initials were isodiametric, darkly stained and some of them were organized in clusters or meristemoids and were presumed to be cambium derivatives. The cells extended into the cortex and the rupturing of the epidermal layer might have been an early root primordium development.

Lateral root primordia were observed on the third day after primary root development (Fig. 32c, d). The meristematic derivatives divided and formed a protuberance of cells of three to four layers, which penetrated into the root cortex.

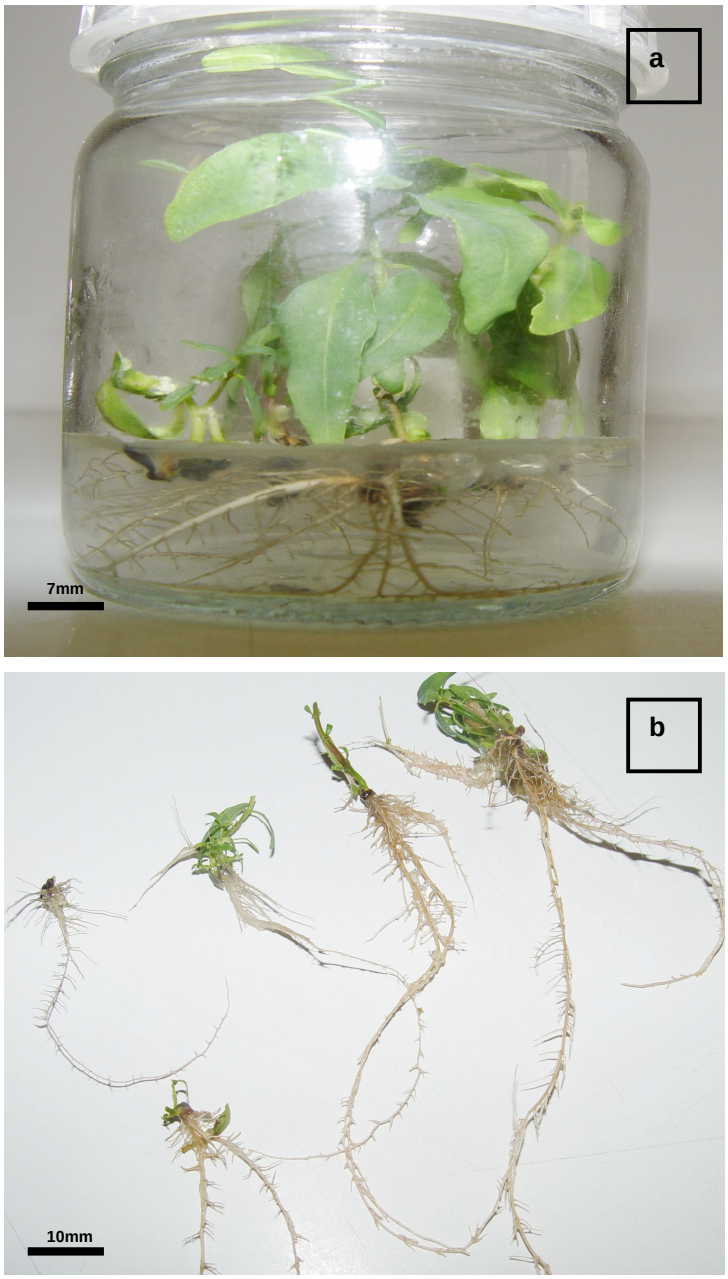


Figure 27: *In vitro* adventitious rooting of *Eucalyptus grandis* x *urophylla.* (27a) Adventitious roots developed in the root induction medium after four weeks. (27b) Rooted shoots after 8 weeks.

3.3.2 Structure of adventitious roots formed from callus differentiation but which lacked vascular connection to shoots

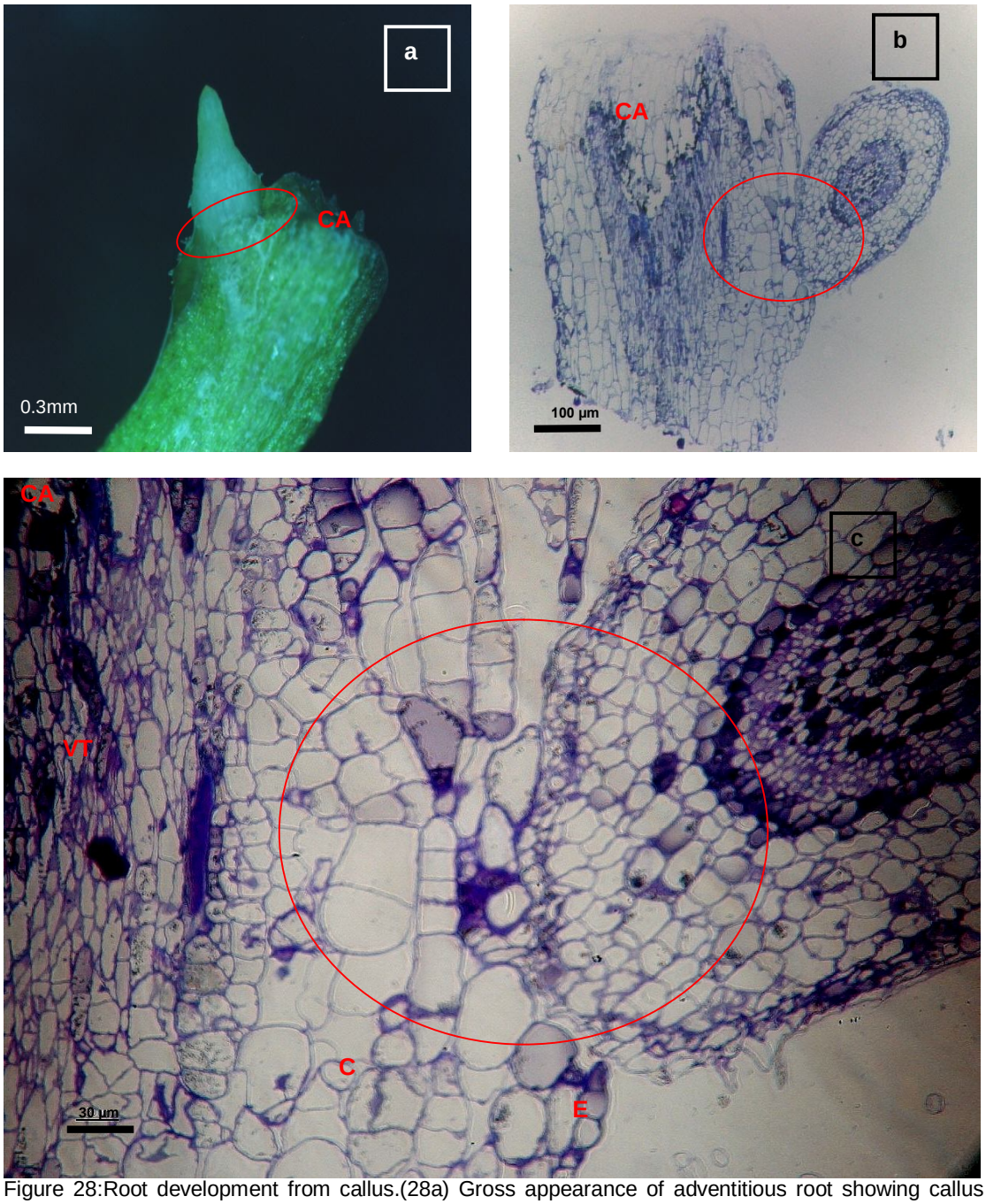


Figure 28:Root development from callus.(28a) Gross appearance of adventitious root showing callus (CA).(28b) Longitudinal section through a root.(28c) No continuous vascular connection formed between shoot vascular tissue and the root (areas shown by circles). Epidermis (E), Cortex (C).

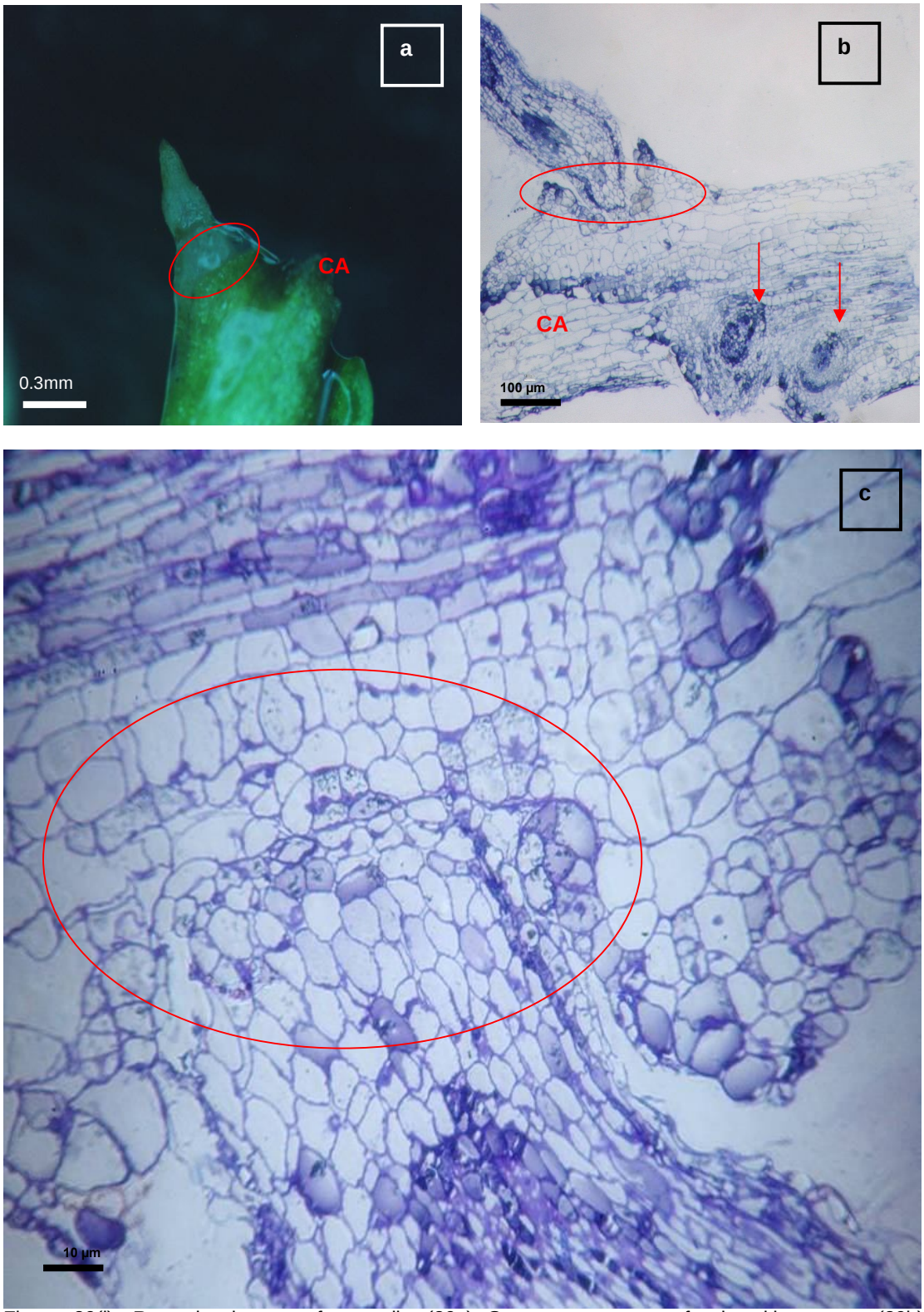


Figure 29(i): Root development from callus.(29a) Gross appearance of adventitious root. (29b) Longitudinal section through a shoot and root, with two distinct developmental stages (root formed from callus (CA) and initiation of root initials from cambium and parenchyma tissue).(29c) Absence of vascular connection between the newly formed root and shoot (shown by circles).

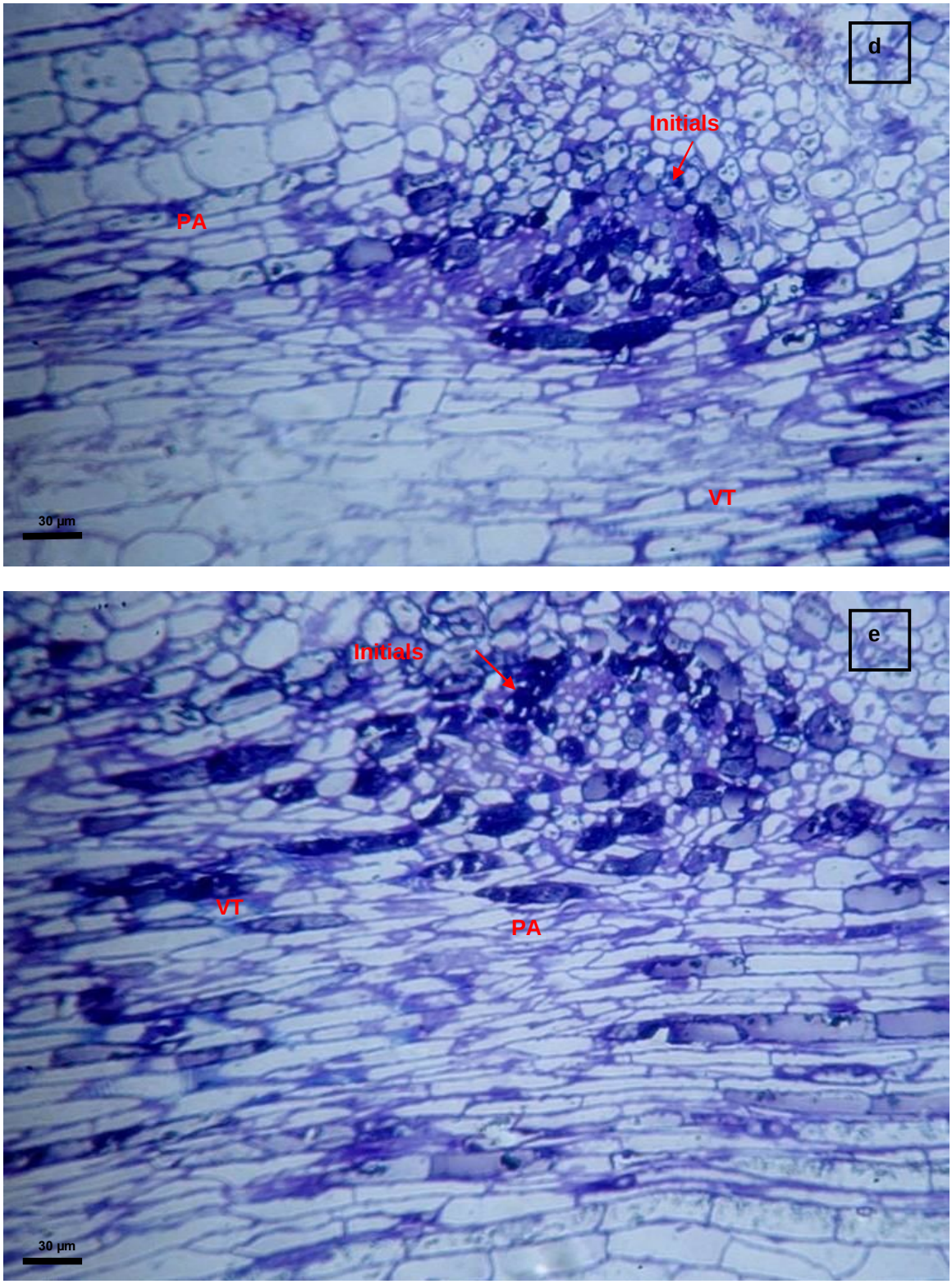


Figure 29(ii): (29d, 29e) Root initial differentiation (arrows) forming from vascular cambium of shoot. Parenchyma cells (PA), Callus (CA) , Vascular tissue (VT).

3.3.3 Structure of roots induced from cortical parenchyma and vascular cambium with a shoot/root vascular connection

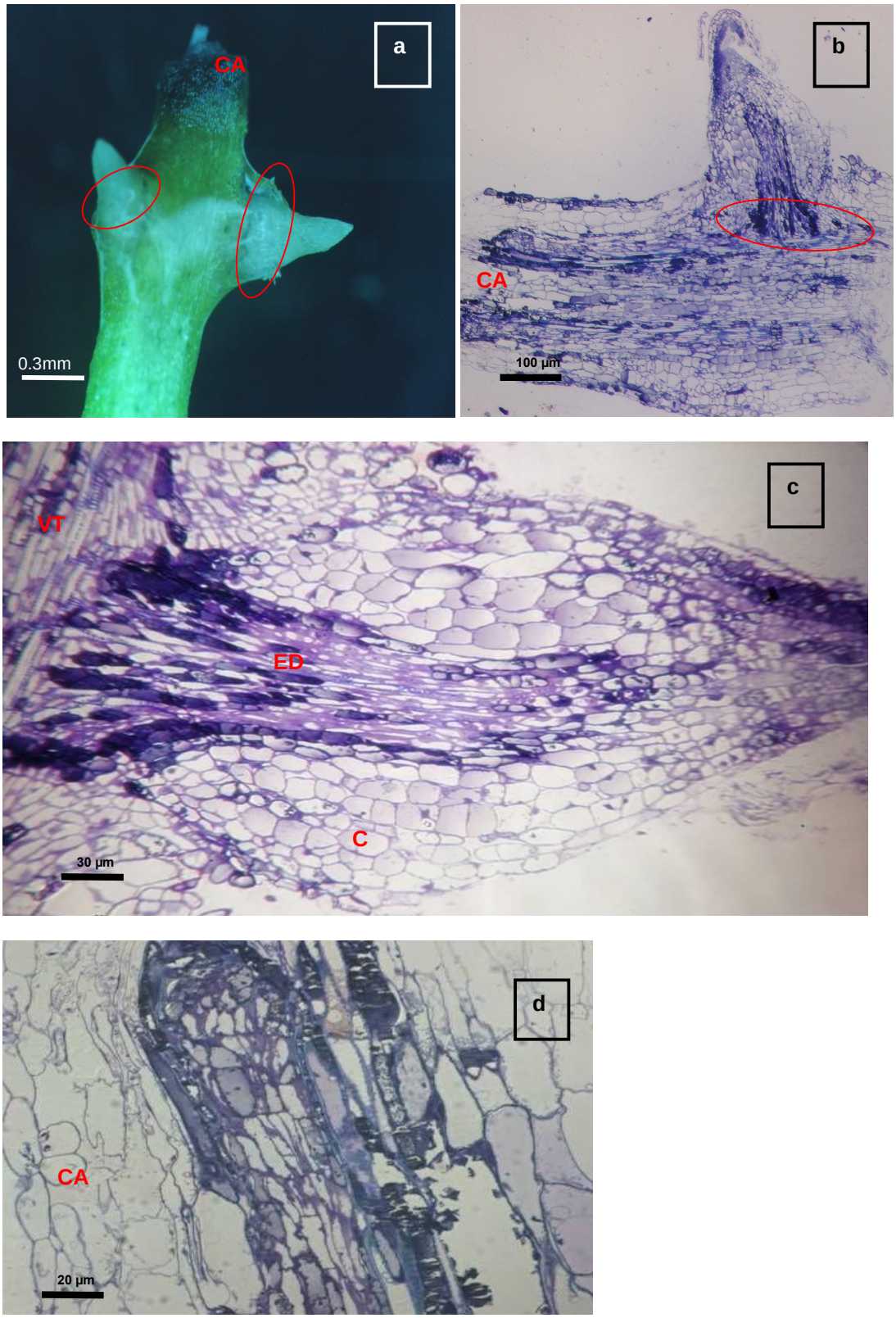
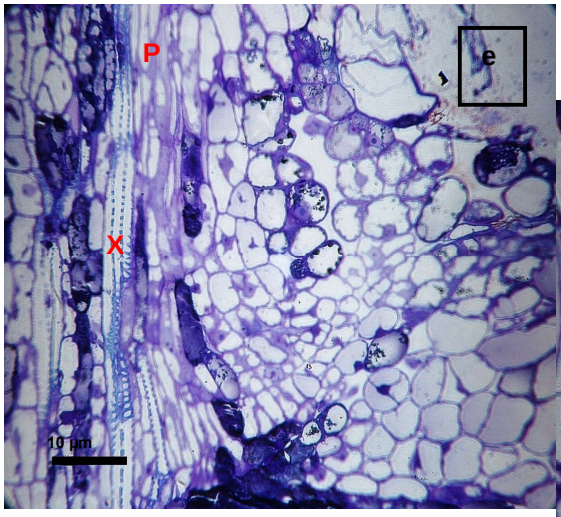
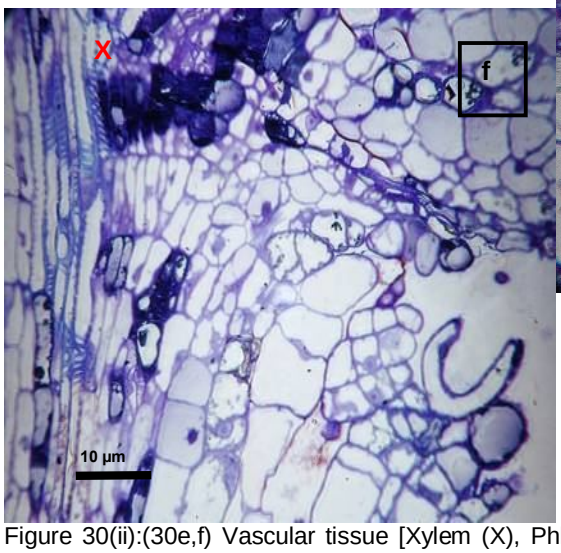


Figure 30(i): Root development from cambium and parenchyma cells of the shoot.(30a) Gross appearance of adventitious roots.(30b) Longitudinal section through shoot and root. (30c) Structure of root with the vascular tissue (VT).(30d) Callus (CA).





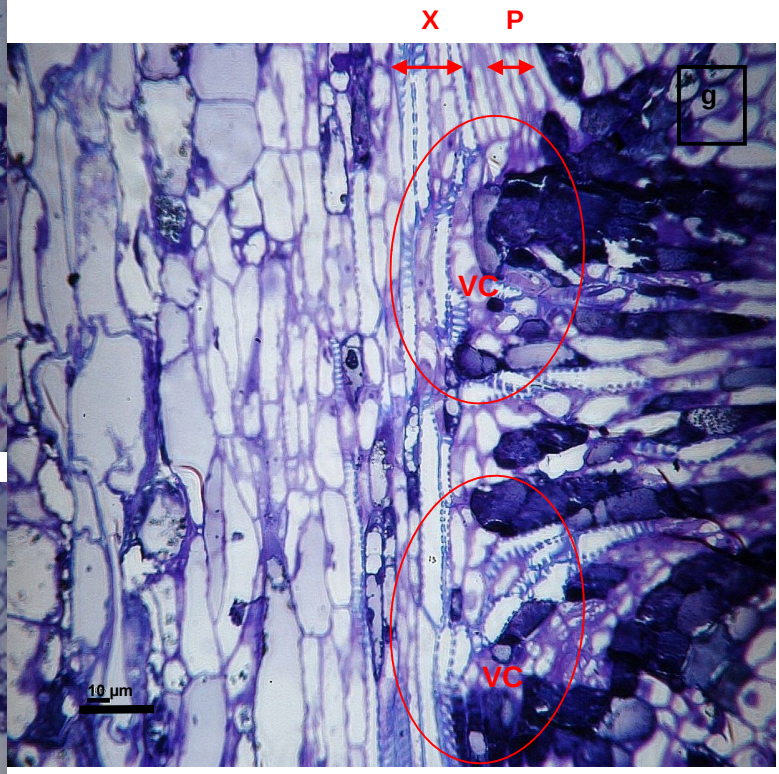
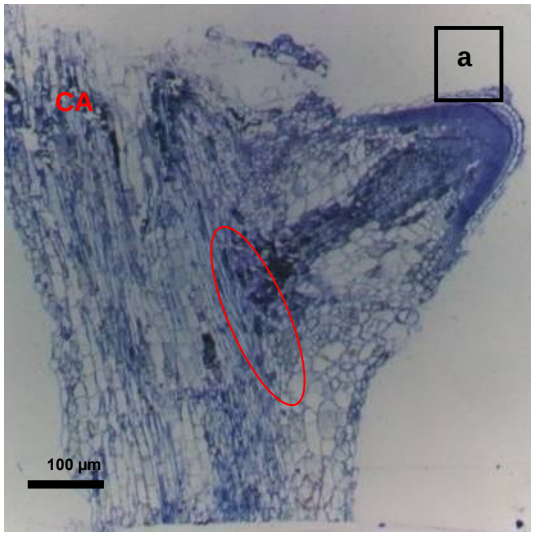


Figure 30(ii):(30e,f) Vascular tissue [Xylem (X), Phloem (P)] of the shoot connected to the root.(30g) Vascular connection (VC) between the shoot and root (circled areas) .



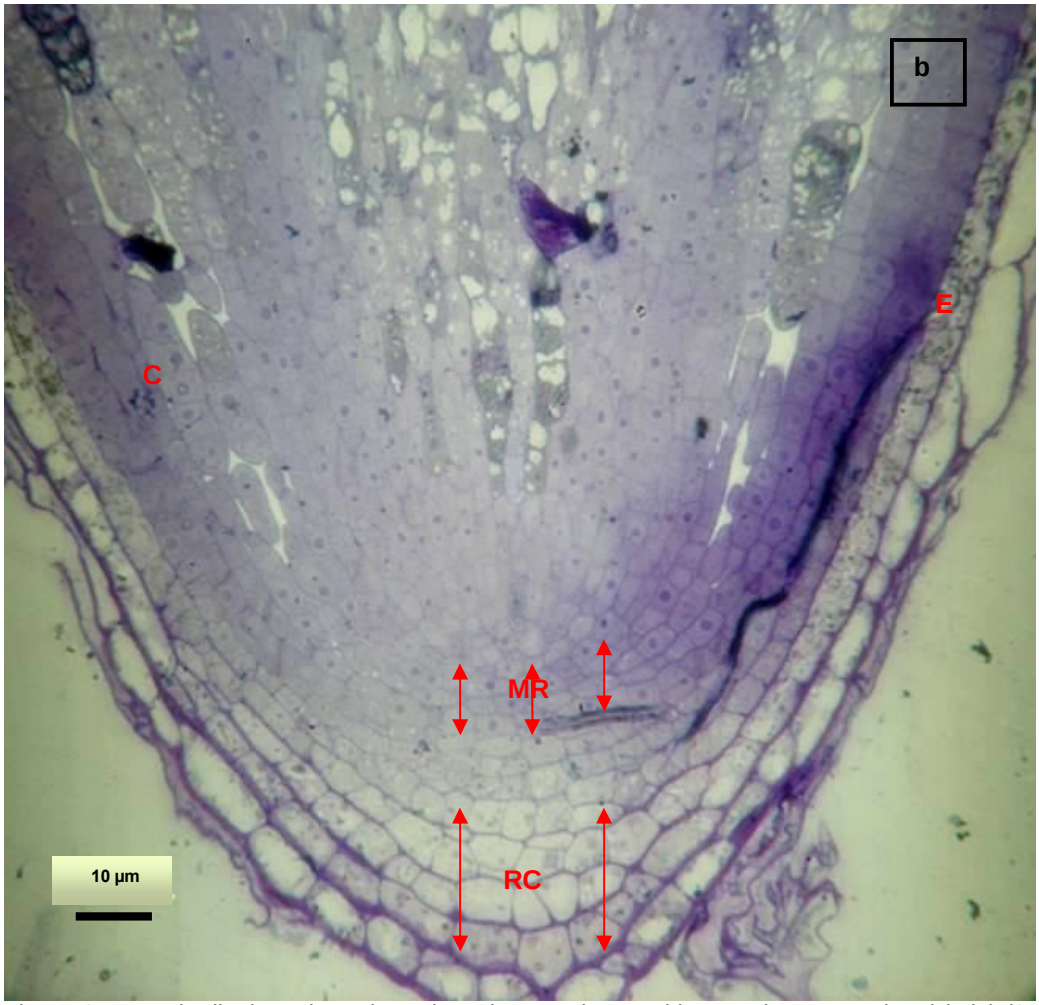
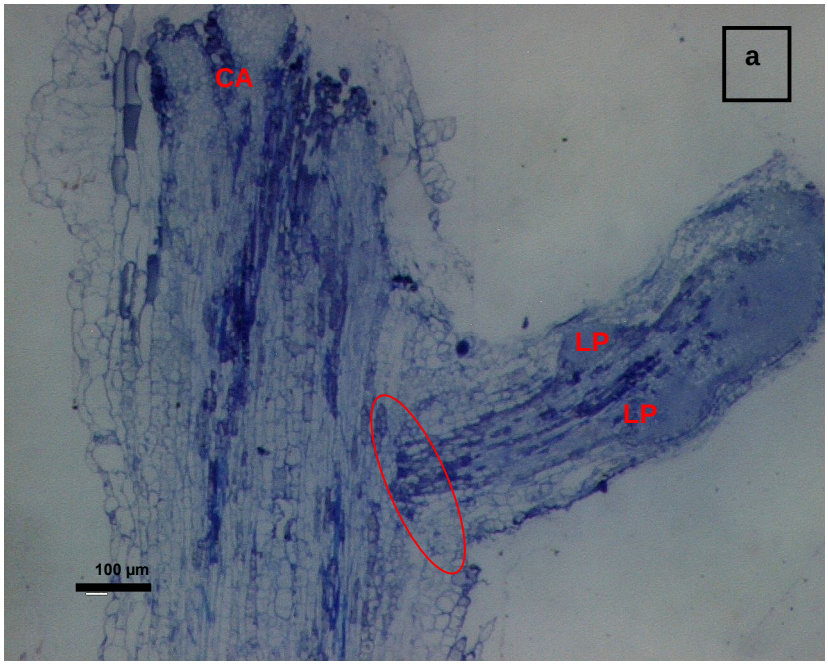


Figure 31: Longitudinal sections through a shoot and root with vascular connection (circle).(32b) The structure of root comprised Meristematic area (MR), Cortex (C), Epidermis, Root cap (RC).



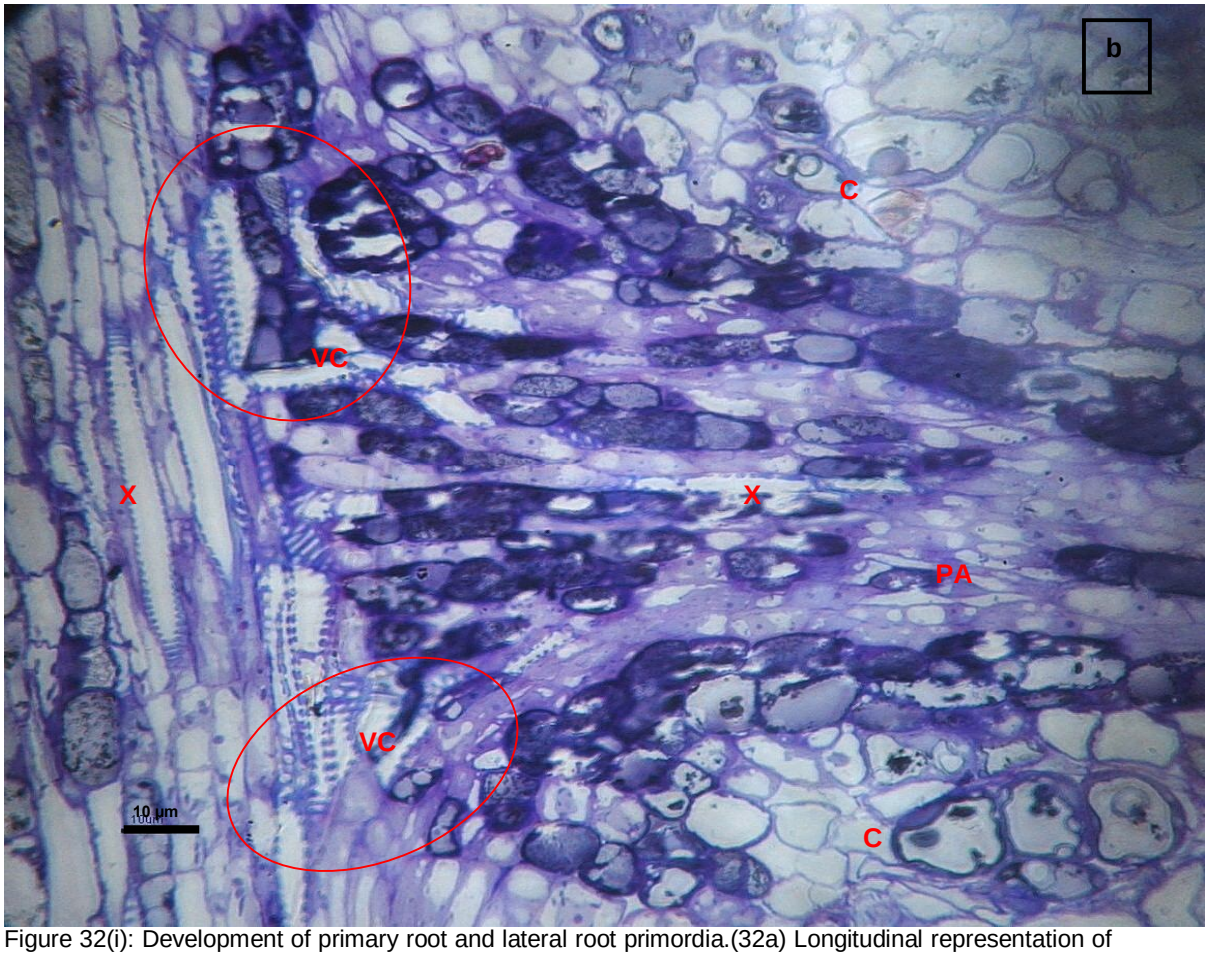
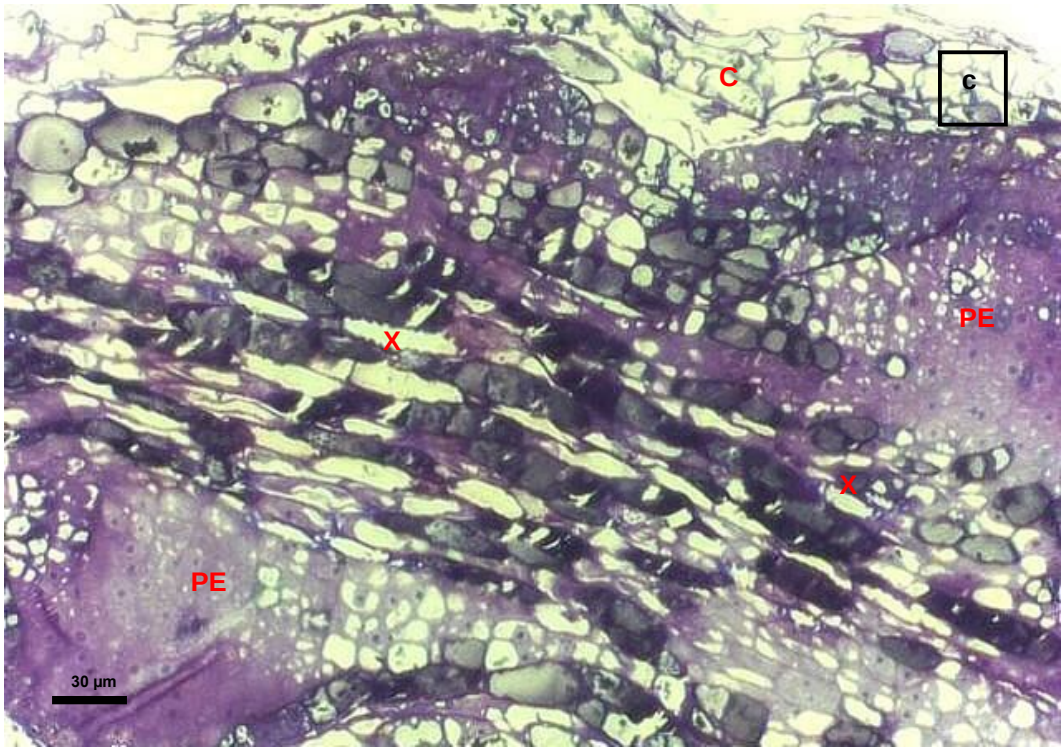


Figure 32(i): Development of primary root and lateral root primordia.(32a) Longitudinal representation of a primary root showing early stage of lateral root initiation.(32b) Vascular connection (VC) between the shoot and root (circled areas).Xylem (X), Cortex (C), Parenchyma (PA).



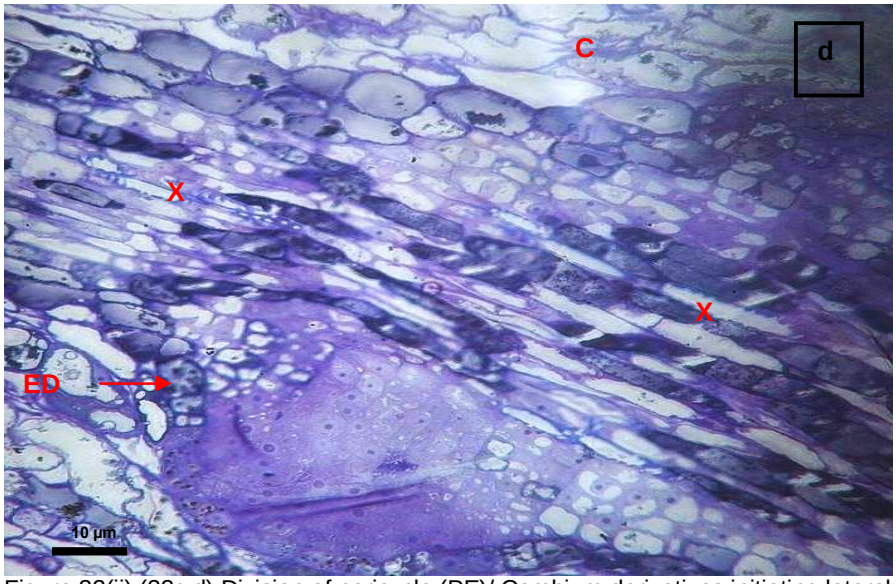
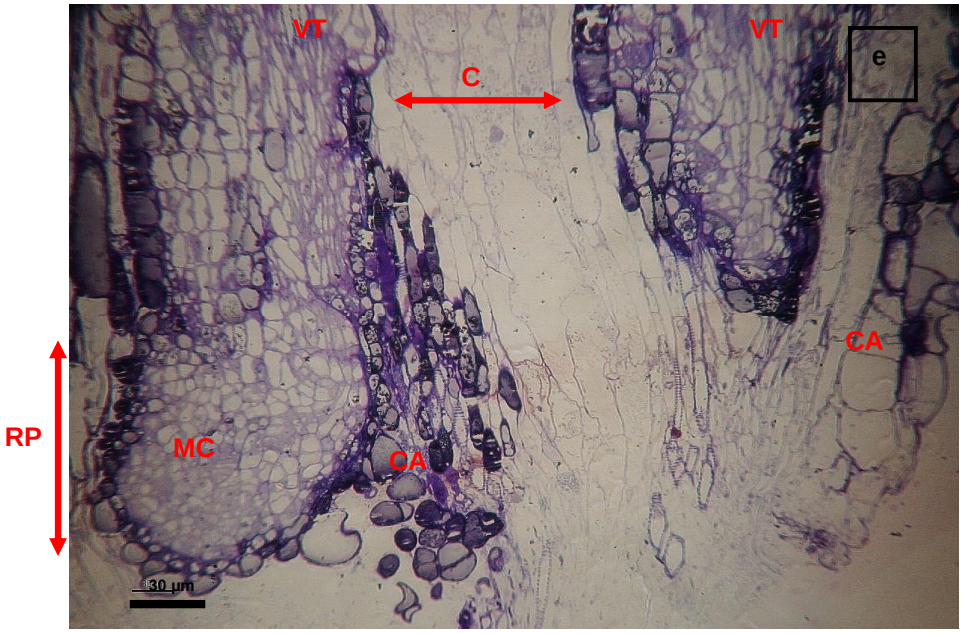


Figure 32(ii).(32c,d) Division of pericycle (PE)/ Cambium derivatives initiating lateral root primordium. Endodermis (ED), Cortex (C).



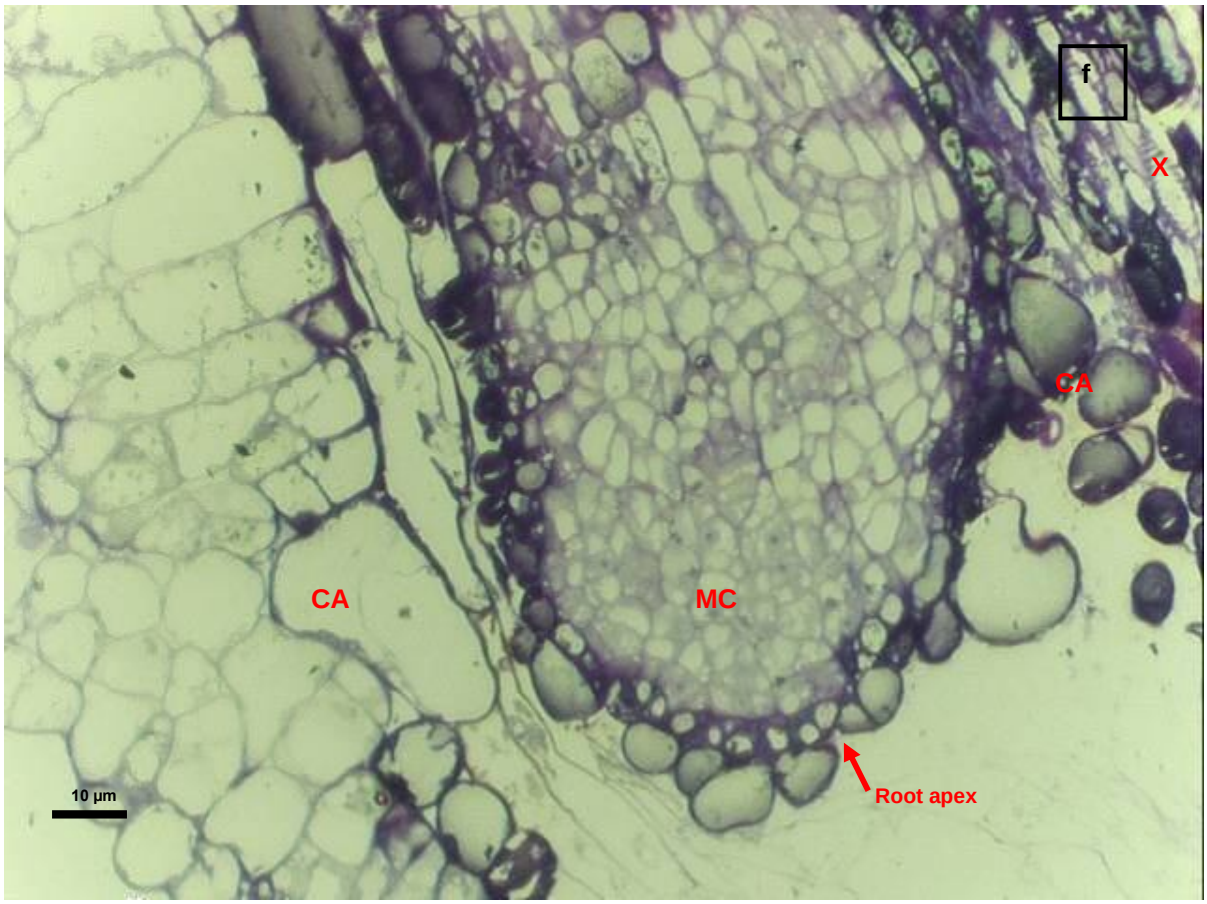


Figure 32(iii):(32e,f) Mitotic division of cells associated with callus (CA),Vascular tissue (VT) with xylem and cortex (C) of the stem. Root primordium (RP) closely associated with xylem (X). Meristematic cells (MC),

3.3.4 SUMMARY

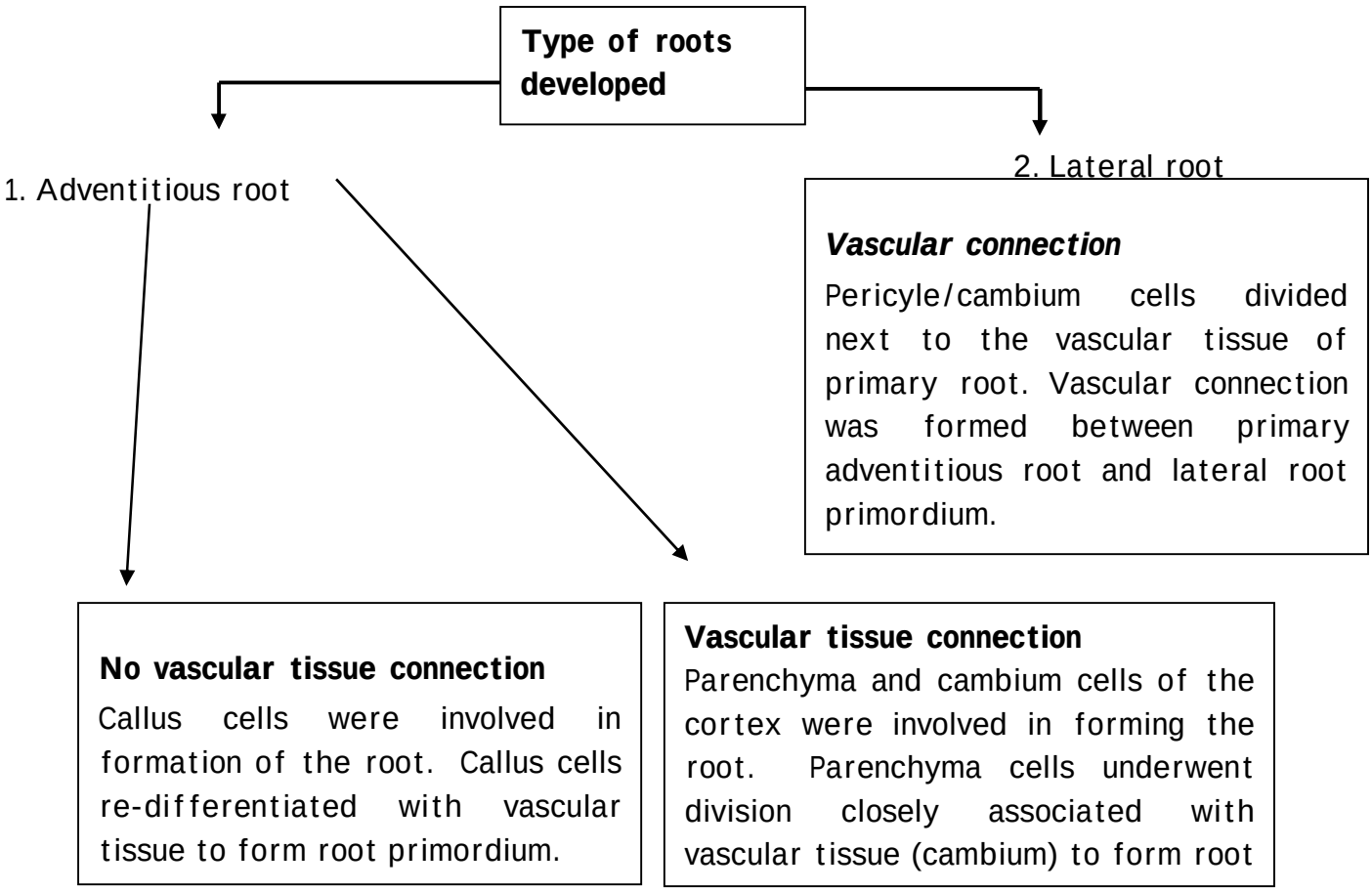


Figure 33: Summary of adventitious roots formed from shoots during rooting of *Eucalyptus in vitro*.

3.4 Acclimatisation

The rooted shoots were first established in rooting mix (2 perlite: 1 coir) for four weeks (Fig. 34). The plants were watered with 1/3 MS salt. After four weeks the plants were transferred to autoclaved sand, and MS salt (4.42 g/L) was supplied to the plants three times per week for a further four weeks and 70% of these plants survived and 30% wilted. The plantlets that survived were then grown in the greenhouse (Fig. 34).



Figure 34: Acclimatised plantlets of *Eucalyptus grandis* x *urophylla.*

3.5 Cryostorage

3.5.1 Drying and cryoprotection

Callus was pretreated in two ways: (1) dried over silica gel for 20 minutes and then exposed to cryoprotectants (dimethylsulfoxide and sucrose) and (2) first exposed to cryoprotectants and then dried. The water content of callus that was not exposed to any drying and treatment was 77.62%. After the first treatment (1) the material had a water content of 56%, whereas after the second treatment the water content was 49% and significance difference was observed between the two treatments. The control callus that was only dried had a water content of 44% (Fig. 36).

3.5.2 Viability and regrowth prior to freezing

Callus (95%) that had been exposed to treatment 1 was viable and 22% callus formed shoots (Fig. 36). Callus that was cryoprotected and then dried (treatment 2) had 100% survival with 20% shoot growth. The drying treatment alone resulted in 92% survival but only 8% shoot growth (Fig. 36).

3.5.3 Viability and regrowth after freezing

3.5.3.1 Rapid freezing

After retrieval from rapid freezing in liquid nitrogen and rapid thawing the callus had turned dark brown. Callus was immediately cultured on fresh medium and regrowth was followed for four weeks. Shoot-buds did not develop and the calli remained dark brown for both treatments (1 and 2) and the control (no treatment). Tetrazolium testing at the end of the regrowth period (four weeks) showed that none of the callus was viable (Fig. 36). No significant difference was observed between the treatments (1 and 2) and control as none of the material was viable.

3.5.3.2 Slow freezing (two-step freezing)

Callus that was frozen slowly also turned dark brown (Fig. 35). As with the fast freezing the calli in this treatment showed no sign of shoot bud formation

after the four recovery weeks, however the tetrazolium test showed that 55% (treatment 1) and 52% (treatment 2) of calli were viable (Fig. 36).

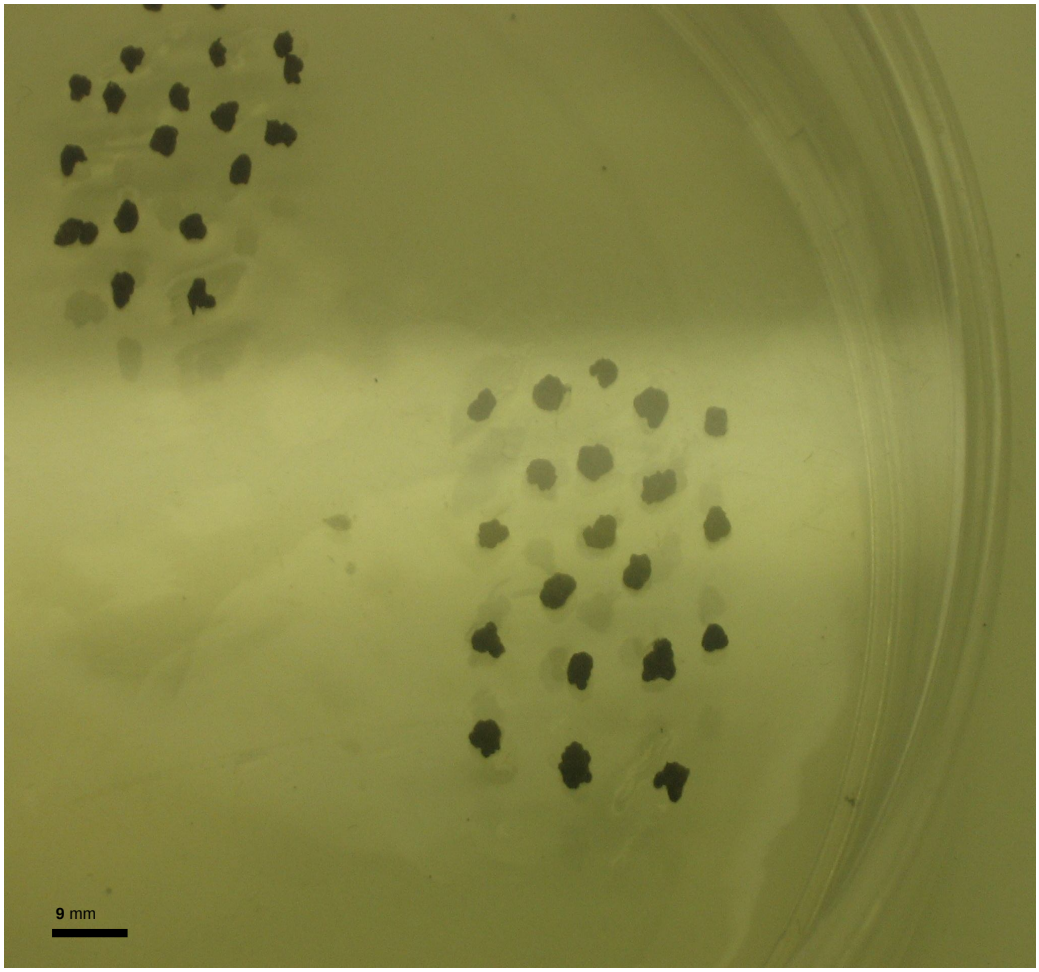


Figure 35: Callus material after slow freezing treatment.

Treatment /Protocol		Parameters tested	Results (% callus)
T test results 1 1. Callus 2. Dry (20 mins) 4. Water content and viability testing		Water content Viability after drying Regrowth on medium	44 ± 21 92±7 8±5
T test result 2 1. Callus 2. Dry (20 mins) 3. Treatment wit cryoprotectants 4. Water content 5. Freeze 6. Regrowth	Slow freezing →	Water content at point of freezing Viability after drying Regrowth on medium (after 4 weeks) Viability after freezing Regrowth on medium (after 4 weeks)	56± 22 95±5 22±3 55±5 0
	Rapid freezing →	Water content at point of freezing Viabilty after drying Regrowth on medium (after 4 weeks) Viability after freezing Regrowth on medium (after 4 weeks)	56±22 95±5 22±3 0 0
T test results 3 1. Callus 2. Treatment with cryoprotectants 3. Water content 3. Dry (20 mins) 4. Water content 5. Freeze 6. Regrowth	Slow freezing →	Water content at point of freezing Viability after drying Regrowth on medium (after 4 weeks) Viability after freezing Regrowth on medium (after 4 weeks)	49±18 100±0 20±4 52±3 0
	Rapid freezing →	Water content at point of freezing Viability after drying Regrowth on medium (after 4 weeks) Viability after freezing Regrowth on medium (after 4 weeks)	49±18 100±0 20±4 0 0

Figure 36: Water content, viability and regrowth of callus prior to, and after, freezing. n=60 ± Standard deviation. Treatment 1(T test result 2), Treatment 2 (T test result 3).

4. DISCUSSION

Three distinct regions/zones of calli *viz.* crystalline, nodular shiny or glassy (either yellow/white/brown) and dark patched calli were recognizable in a callus mass in this study. It was noted that the regions described could form part of one callus mass or were found in different calli. The crystalline callus usually covered the surface of the brown, yellow or white callus. Bandyopadhyay *et al*. (1999) described callus of *Eucalyptus nitens* and *Eucalyptus globulus* derived from hypocotyl tissue as being nodular and compact, creamy to brownish and cotyledons as nodular,creamy,white or pale brown which were some of the similarities indicated in this study (nodular brownish callus). Blakeway *et al*. (1993) characterized the regions of calli that were induced from cell suspensions cultures of *E. grandis* and *E. grandis x calmadulensis* into three classes: Type I (cream/white and soft, cream/white and nodular), type II (brown and soft, and nodular) and type III (glassy/transparent and soft, glassy/transparent and nodular). Microscopy of type I callus revealed actively dividing "embryonic cells" or cells containing large nuclei. Type II callus comprised of small embryogenic cells containing large nuclei, whilst type III callus had irregular shaped and large embryogenic cells that contained small nuclei and large vacuoles. Some similarities in the structure of the *Eucalyptus grandis* x *urophylla* callus were found when compared with the study of Blakeway *et al*. (1993) even though a different approach was used to induce the callus. According to the Blakeway *et al.* (1993) descriptions, in this study, the Type I and II was resemblance of the nodular shiny or glassy callus that was brown, yellow or white and the crystalline callus (the irregular large size of the cells) as type III. The results in this study indicated that some similar characteristics of Eucalyptus.

In the present study, the histological pattern of growing calli that gradually formed throughout the process included two types of cells, morphogenic or meristematic-like, and nonmorphogenic cells.. After 15 days of callus induction and culture, morphogenic cells were distinguished as small actively dividing cells that formed in clusters, were densely packed and contained visible nuclei and nucleoli (Figs. 4e,f). These cells were close to the

developing areas of vascular tissue. These are presumed to be equivalent to the embryogenic and non-embryogenic cells described by Blakeway *et al*. (1993). It was reported that primary vascular tissue is produced from procambium during the differentiation of meristematic cells and is important in the primary development of plant organs (Ye, 2002). Popielarska *et al*. (2006) induced callus from seed endosperms and observed two distinct types of calli; morphogenic and nonmorphogenic. The morphogenic callus that was produced from the endosperm tissue had large rounded cells that formed in groups of different sizes. Those cells proved to have the capacity to form shoots. Nonmorphogenic cells were long and loosely arranged.

In the present study the tissue that contained a central gap surrounded by meristematic-like cells were observed in cross section (Figs. 4e, 7b, 11e). These cells were assumed to be the initiation or presence of oil glands. Oil glands with similar organization of cells (e.g. visible central cavity) developed in *Eucalyptus globulus* Labill. callus and hypocotyls during bud regeneration (Azmi *et al*., 1997). Azmi *et al*. (1997) reported that shoot meristems and buds were induced next to the differentiating oil glands.

In this study, the callus developed nodular structures within 22 days of culture. Cells that formed in the nodular areas of callus of *Eucalyptus grandis x urophylla* had small tightly packed morphogenic or meristematic-like cells while the nonmorphogenic cells appeared large and irregular (Fig 7 and 8). Shoot-bud like structures occurred on the periphery of nodular calli (7a&b, 8a&b). On day 29 of culture, vascular tissue connection to morphogenic clusters of cells in different parts of the callus was observed. The connection formed a chain of vascular system (Fig. 10a). The parts where the vascular system was connected to the clusters of cells (Figs. 10b,c,d,e) and some of the callus did not show the vascular chains but only small areas of morphogenic cells dividing close to the vascular tissue Figs 11c,d,e). Chen and Galtson (1967) described vascular nodules in callus as a mass of cells consisting of parenchyma and vascular tissue and these cells were responsible for early shoot development. In this study the areas where the group morphogenic cells were associated with the vascular tissue might have

been the areas of differentiation of nodular bulges that were to develop into shoot-buds and they could also represent an earlier stage of shoot development.

The calli that formed between day 36 to 57 were entirely covered with nodules that bulged out on the surface of the callus (Figs. 12-19). The cells of these nodules were either entirely morphogenic/meristematic (Figs. 15,18) or the nodules had areas of differentiation that were composed of morphogenic and vascular tissue (Figs. 13;14;17;20). The nodular structures seemed to be the active zones of shoot meristem initiation and shoot-bud development. The nodular callus regions were resemblance of Type I and II callus [according to description of Blakeway *et al*. (1993)] which was brown, yellow or whitish and sometimes possessed dark patches. The increased crystalline layer on the surface of calli was comprised of nonmorphogenic cells. In this study callus that had a red pigment also formed but in low frequency (16e;18b) and greenish pigmented areas were also identified on surface of callus (days 22-43). A similar observation of red and green zones was characterized in the callus of *E. nitens* and *E. globulus* (Bandyopadhyay *et al*., 1999) and *E. camaldulensis* (Dibax *et al*., 2005). Bandyopadhyay *et al*. (1999) made assumptions that the red pigment that occasionally occurred on callus induced from cotyledons and hypocotyls was due to the production of anthocyanins. In *E. camaldulensis* red and green callus that developed from the petiole of cotyledonary leaves was regenerative and developed shoot-buds and furthermore the diversity of colours in the callus was also associated with production of anthocyanins (Dibax *et al*., 2005). This indicates in the present study that red and greed pigmented callus can be associated with or have the potential to develop into shoot-buds.

The pathway of callus initiation and shoot development observed in the present study can be described as follows: the callus was induced from proliferation of parenchyma cells which induced morphogenic and nonmorphogenic cells and vascular tissue. Morphogenic cells proliferated in association with vascular tissue to collectively form vascular nodular structures. The nodular structures were characterized by closely packed

meristematic regions. The presence of vascular nodules at the periphery of the callus signified the initial stages of differentiation and early stages of initiation of shoot- bud like structures. The process of indirect organogenesis involves four different developmental steps, dedifferentiation, induction, differentiation and organ formation (Schwarz and Beaty, 2000). Dedifferentiation involves reversible development of mature cells. This is followed by induction of undifferentiated parenchymatic callus cells which undergo differentiation. Callus differentiation can be associated with the formation of meristematic cells. These cells form meristemoids, or nodular structures that can continue dividing and eventually differentiate into specialized structures or tissues (e.g. xylem and phloem) that are capable of forming a functional organ (Cassells, 1979; Romberger *et al*., 1993; Chawla, 2002).

In other *Eucalyptus* plants, shoot-buds formed from protuberance structures that originated from leaves, nodes and internodes (Hervè *et al*., 2001). In the case of *Passiflora* species, hypocotyls were used in initiating shoot-buds both directly and indirectly. Cell proliferation occurred next to the cut surfaces of explants from the parenchyma cells based in cortex or pith and developed meristemoids or callus (Fernando *et al*., 2007). The meristemoids developed shoot-buds or protuberances which eventually formed shoots. In Black pepper, callus was induced from leaf explants and the proliferation of parenchyma cells was observed next to the vascular bundles of mesophyll (Sujatha *et al*., 2003). The cells became meristematic and the procambium was induced during differentiation and formed vascular elements such as tracheids at the base of the callus. Clumps of meristematic initials developed into vascular nodules which formed shoot-buds (Sujatha *et al*., 2003). Somatic embryos in *Solanum melongena* were formed directly from parenchymatic perivascular cells or parenchymatic vascular tissue. Proembryos that were generated via parenchymatic vascular tissue had to first form indeterminate meristematic cells which led to expansion of groups of proembyos. Masses of proembyos and indeterminate meristematic cells redifferentiated into tracheary elements then vascular bundles (Tarre *et al*., 2004). Cells and tissues described in this study resemble some similar

characteristics of shoot initiation and development from callus callus of the leaf, nodes explants and internodes explants and embyos described (Hervè *et al*., 2001; Sujatha *et al*., 2003; Tarre *et al*., 2003 and Fernando *et al*., 2007).

At the end of the eight weeks 39% of the calli regenerated shoots. Hajari *et al*. (2006) achieved 47% of shooting for *Eucalyptus grandis x urophylla* (GU1) and 13% for *Eucalyptus grandis x urophylla* (GU2) clones. The total shoots that produced roots after four weeks of culture was 74% while Hajari *et al*. (2006) had a total root production of 83%. The difference on the regeneration of shoots and roots could have been the choice of explant used, as Hajari *et al*. (2006) initiated callus from shoot stems without axillary buds.

The fundamental structure of a root comprises an epidermis, cortex, endodermis and the inner layer of vascular and parenchyma tissue. The tip of the root is covered by a root cap. Cells that form part of a root cap are the columella and lateral root cap cells (Sussex, 1989; Dolan *et al,* 1993; Schiefelbein *et al.*, 1997, Wenzel and Rost, 2001). Some of these structures were recognized in the roots that developed from the shoots of *Eucalyptus grandis x urophylla* in this study (Figs. 31c) which represented a true structure of a primary root.

In this study, it was established that 50% of shoots (initiated from callus) developed a vascular connection between the shoots and the newly formed roots. The roots that formed a connection to the shoots seemed to be induced from the cambium parenchyma of the shoot. Groups of cell initials that were heavily stained were identified in the cambium region (Figs. 29d,e). These cells appeared to be dividing. The vascular connection (Figs. 30-33) can be presumed to have been induced from cambial parenchyma cells that resulted in the differentitaion of vascular tissue connecting to the new root primordia. Three significant consecutive phases can be described in adventitious rooting; the induction, initiation and expression phases. Biochemical and molecular pathways can be identified in the induction phase of adventitious roots (Kevers *et al*., 1997). In the initiation phase of adventitious rooting, a group of root meristem forms by cell division while the

expression phase is distinguished by root primordia and the emergence of the new root (Kevers *et al*., 1997; De Klerk, 2002). In stems of woody plants adventitious roots can be formed either directly near the vascular cambium (Esau, 1965; Altamura, 1996) or indirectly from division of undifferentiated callus (Altamura, 1996). Both direct and indirect patterns can form meristemoids, root primordia and roots (Altumura, 1996). This involves the vascular tissue that differentiates into shoots, and the tissues that usually form part of this cell differentiation can be parenchyma cells including parenchymous-like cells of callus (Soh and Bhojwani, 1999). These parenchyma cells appear meristematic with a well-characterized nucleus with nucleoli, such as those of the vascular cambium (Soh and Bhojwani, 1999). Baltierra *et al*. (2004) described the histological processes of *in vitro* rooting in *Eucalyptus globulus.* Shoots were cultured on rooting medium containing KNO_3, indole-3-butyric acid (IBA). Callus de-differentiation was observed at the base of the stem on the third day of rooting. Parenchyma cells in the shoot divided and formed group of meristematic cells associated with vascular nests which initiated root primordia. The entire rooting process took 12 days and roots were initiated.

Shoots that failed to form a vascular connection to the initiated roots showed that callus formation at the base of the stem and root primordia were induced from this callus (Figs. 28c, 29c). An irregular callus was associated with the primordia which was comprised of cells that were isodiametric with distinct nucleoli (26c, 32c). It is essential that roots develop from vascular tissue of the shoot rather than the callus because this can be a major drawback in the establishment of plants (Soh, *et al*., 1999). Basal calli formed on shoot stems of some *Eucalyptus grandis* hybrids; as a result this caused inadequate rooting (Le Roux and Van Staden, 1991a, 1991b, Jones and Van Staden, 1994). Similarly, in the rooting of some conifers, it was found that the plants that developed roots from the callus were unable to survive transplantation (Thorpe, 1984). Grout and Aston (1977) reported that inefficient *in vitro* rooting of caluliflower reduced the uptake of nutrients and water-signalling to the shoots and resulted in poor plant acclimatization or mortality. Metivier *et al*. (2007) observed the same phenomenon in rooted microshoots of *Cotinus*

cogygria, a woody ornamental plant. Adventitious roots formed from callus at the base of the stem were distorted and contained highly vacuolated cortical cells and broke easily during transplantation (Metivier *et al*., 2007). The results in this study indicated that the roots that were initiated from the callus did not survive acclimatisation after rooting process as 30% of the plants died.

In this study, the lateral root primordium was initiated from differentiation of pericycle cells (Figs. 32c,d). The developed cells were positioned next to the xylem vessels. The position of xylem vessels can be related to the direct link that will form between the parent root and the lateral root (Figs. 32c,d). Cells and tissues responsible for initiation and development of lateral roots are pericycle cells, xylem, phloem or vascular parenchyma found the in parent root (Esau, 1977; Laskowski *et al*., 1995). The cells undergo a series of division periclinally and expand into meristematic protuberances that will ultimately rupture through the cortex and endodermal layer into a fully developed root (Blakely and Evans, 1979). Such differentiation creates a vascular connection between the parent root and lateral root (Esau, 1977). Lateral roots elongate and improve the absorptive area of the root system and thus initiate secondary growth (Trigiano and Gray, 2005). In the developmental process of lateral root formation in cork oak, lateral roots were primarily induced from dividing cells opposite the xylem strands (Verdaguer *et al*., 2000) as was observed in the present study. Verdaguer *et al*. (2000) initially observed proliferation of pericycle cells periclinally and endodermis expanded opposite the xylem strands which eventually formed a dome of cells. The lateral roots induced were linked by xylem and phloem strands through pericycle derivatives that protruded through the cortical tissue (Verdaguer *et al*., 2000).

The histological studies carried out with the regenerating callus at different stages of growth aided in choosing the potential stage in the freezing of callus. Callus that was iniated for (22 days) was used as starting material for cryopreservation of *Eucalyptus grandis x urophylla.* On day 22, the cells of the callus were at an early stage of differentiation. When cryopreserving plant cells or tissues the choice of material (including size and type) contribute to a

successful cryopreservation procedure (Reinhoud *et al*., 2000). Plant material that contains small cells is less susceptible to freezing and thawing damage than material comprised of large cells (Nag and Street, 1975; Engelmann, 1991). Hence the choice of day 22 callus that was predominantly meristematic in nature. During freezing the amount of water in plant cells or tissues affects the degree of damage that is caused by ice formation and consequently survival rate (Mycock *et al*., 2004; Volk and Walters, 2006). Vacuoles in the plant cell are filled with water that can be freezable. Meristematic cells have smaller vacuoles and therefore have less freezable water (Engelmann, 1991). Callus and cell suspension cultures usually have heterogeneous cell populations and can have large highly vacuolated cells (Withers, 1987; Reinhoud *et al*., 2000). The heterogeneity found in callus can cause some of the cells to be more tolerant to injury than others (Reinhoud *et al*., 2000). The amount of water in plant material such as calli, shoot tips and somatic embryos is therefore reduced via various methods so as to minimise ice crystal damage during freezing and thawing (Reinhoud *et al*., 2000).

In the present protocol the water content was reduced by drying over silica gel for 20 minutes prior to or after treatment with cryoprotectants. The exposure of callus to cryoprotectants for both treatments improved the survival and regrowth after drying when compared with the material that was only dried (Fig. 36). The callus pretreated via the first treatment, although it had a higher water content, resulted in greater shoot production than that subjected to treatment 2 (Fig. 36) and significant differences were observed between the two treatments (Fig. 36). Sucrose was a component of the cryoprotectants solutions used in the present investigation. Stewart *et al*. (2001) achieved post thaw viability when somatic embryos of cassava were pretreated with mixture of sucrose and glycerol. Cryoprotectants can decrease the amount of water in the cells by removing water to the external medium. The cell structures such as the cell membrane can also be retained and protected against damage during drying, freezing and thawing by the use of certain cryoprotectants, e.g. sucrose (Sakai, *et al*., 1991; Bryant *et al*., 2001; Crowe *et al*., 1998; Wolfe *et al*., 2002; Rao, 2004).

Eucalyptus calli were cryostored by both rapid and slow freezing methods. The post thaw viability after slow freezing was 55% (treatment 1) and 51.67 % (treatment 2). There was no regrowth within four weeks after freezing regrowth (Fig. 36). Slow freezing has been successful in cultures of callus and cell suspensions (Kartha and Engelmann, 1994). Examples of successfully slow frozen cultures are embryonic tissue of *Pinus patula* (Ford *et al*., 2000) and callus of *Artemisia annua L*. (Chenshu *et a*l., 2003). When tissues or cells are slow frozen, ice crystals are formed in the extracelullar medium, and water is osmotically removed from the cells to the external medium (Engelmann, 1991; Reinhoud *et al*., 2000). Also during thawing small ice crystals formed during freezing can recrystallise and cause disruption to cell structure (Withers, 1980; Mazur, 1984; Steponkus and Lynch, 1989). In the present study the results indicated that slow freezing rate can be a potential method for freezing of callus than rapid freezing as none of the callus were viable after rapid freezing and thawing (Fig. 36). This was probably due to damage caused by ice crystals inside the cells upon freezing/ thawing. When cells are being frozen, intracellular water can form ice which can destroy the cell structure.

5. CONCLUSION AND FUTURE PROSPECTS

Callus was successfully induced from axillary bud segments of the *Eucalyptus grandis* x *urophylla* hybrid. However the protocol (Hajari *et al*., 2006) did not result in a high production of shoot-buds. In the studies of Hajari *et al*. (2006) the complete shoot stems of *Eucalyptuts grandis* x *urophylla* clone (GU1) were used and they had higher shoot regeneration (GU2 that produced less shoots) than in the current study. Since this protocol had low yield of shoots, this area of study needs to be revisited.

The morphological and histological pathway of organogenesis from callus outlined in this study demonstrated that the nodular, compact, glassy callus was regenerative and had the potential or capability to form adventitious shoots. The morphogenic cells that formed part of this nodular callus formed vascular nodules which reperesented the first signs of organ initiation. It can also be presumed that the vascular elements themselves were also derived from these morphogenic cells.

Plants that are rooted adventitiously have an important role in the establishment of plants for the forestry and horticulture industries (Altamura, 1996; Baltierra *et al*., 2004). However difficulties can arise during the rooting process e.g. failure to root by juvenile or seedling stage, low frequency of root production (Fett-Netto *et al*., 2001) and can result in failure in the establishment of clonal plantations (Baltierra *et al*., 2004). Although a high frequency (74%) of rooting of plantlets was achieved in this study poor, or lack of rooting has been documented in some *Eucalyptus* plants such as *Eucalyptus globulus* (De Little *et al*., 1992) and *Eucalyptus nitens* (Bennet *et al*., 1994). This represents *Eucalyptus grands ix urophylla* a potential rooting response. Results in this study indicated that adventitious roots can be formed from two sources i.e. the callus and from cambium tissues in the stem. The roots that were induced form cambium tissue produced a true vascular tissue connection and a functional root system. The evidence was further supported by the ability of the plantlets to survive post *in vitro* conditions. However, with roots that developed from callus without any vascular

connection to the shoots, 30% of the plantlets did not survive the subsequent acclimatization process. The vascular tissue connection between the shoot system and roots plays a role in transporting essential nutrients, photosynthetic products and water from the root to all parts of the plant (Jesko, 1989; Dettmer *et al*., 2008). The results of this study indicated that the roots that developed were fully functional and that had well-developed vascular connections to the shoots and had an ability to extend or increase the root system (i.e. lateral root formation).

The callus subjected to cryopreservative protocols did not regenerate into shoots and roots after a recovery period of four weeks. This could be due to two reasons. Firstly the callus (50% of the material that was viable (tetrazolium chloride test) may have been too damaged to allow recovery growth and development. Secondly, the experiment could have been terminated too early, that is the material was still undergoing recovery processes and given further recovery time the surviving 50% of the material would have resumed growth. This could be ascertained by future research work which could investigate the ultrastructure of callus subsequent to freezing and also allow a longer recovery period. Additionally future research should further consider the rate of freezing and thawing, the present investigation has given the indication that slow freezing is better than fast freezing and this method can be considered for freezing of *Eucalyptus*. Similarly the age and developmental stage of organogenesis within the callus should be further considered. The age of callus used in this study might have been too young with organogenesis at too early developmental stage to survive the freezing effects.

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