ADVANCES IN EMBRYOGENESIS AND ORGANOGENESIS IN COMMON BEAN (*Phaseolus vulgaris* L.)

By

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.), an important legume in the human diet, is recalcitrant to *in vitro* culture, so the establishment of alternative propagation protocols, including somatic embryogenesis and organogenesis, has been a challenge. In the present work, we evaluated conditions to induce somatic embryogenesis and organogenesis from explants of seedlings of two F3 interspecific bean hybrid lines. In order to induce embryogenic callus, different types of explants were cultured under concentrations of 0, 0.01, 0.05 and 0.1 mg/L of 2,4-D in presence of 1mg/L of BA or at 29°C. Hypocotyls were the type of explant showing the highest production of nodular calli, and 0.1 mg/L of 2,4-D was the most favorable concentration. Treatments to induce maturation of nodular callus included air-drying, transfer from solid to liquid medium, culture with $AgNO_3$ and combinations of these factors. Air-drying and transfer liquid medium (with or without $AgNO_3$) enabled the development of heart-shaped or elongated embryo-like structures within calli. Organogenesis was also induced in buds from epicotyls, which were subsequently transferred to two treatments to proliferate: 0.5mg/L of BA and 2.25mg/L of BA with 1.7mg/L of $AgNO_3$. The combination of 2.25mg/L of BA with 1.7mg/L of $AgNO_3$, and 1.5 mg/L GA_3, was used for the induction of bud elongation. Proliferation of buds was achieved with 2.25mg/L of BA and 1.7mg/L of $AgNO_3$. Combinations of BA, $AgNO_3$, GA_3 and ANA were tested to shoot rooting, but were ineffective. To study the structure of calli by scanning electron microscopy, chemical fixation and cryofixation were carried out. Cryofixation treatments included rapid

freezing, slow freezing and slow cooling. Although none of the treatments effectively preserved the calli, some observations and inferences could be made about the structural organization of calli.

RESUMEN

La habichuela (*Phaseolus vulgaris* L.), leguminosa importante en la dieta humana, es difícil de cultivar *in vitro*, por lo cual el establecimiento de protocolos alternos de propagación de habichuela como embriogénesis somática y organogénesis han sido un reto. En el presente trabajo se evaluó las condiciones para inducir embriogénesis somática y organogénesis a partir de explantes de plántulas de habichuela de dos líneas F3 híbridas interespecíficas. A fin de inducir callo embriogénico, diferentes tipos de explantes fueron cultivados en tratamientos con concentraciones de 0, 0.01, 0.05 y 0.1mg/L de 2,4-D en presencia de 1mg/L de BA o mantenidos a 29°C. Explantes de hipócotilo mostraron una mayor producción de callos nodulares en tratamientos con 0.1mg/L de 2,4-D. Se estudiaron tratamientos para maduración de callos nodulares como secado al aire libre, transferencia a medio líquido y cultivo con nitrato de plata ($AgNO_3$), solos o en combinación. El secado al aire libre y medio líquido en presencia o no de $AgNO_3$, permitió obtener estructuras similares a embriones en estado de corazón y torpedo en el interior de los callos. Organogénesis fue inducida en yemas procedentes de epicotilos y transferidas secuencialmente a dos tratamientos para proliferación: 0.5mg/L de BA y 2.25mg/L de BA con 1.7mg/L de $AgNO_3$. La combinación de 2.25mg/L de BA con 1.7mg/L de $AgNO_3$ y 1.5 mg/L GA_3, se uso para el alargamiento de las yemas. La proliferación de múltiples yemas se logró con 2.25mg/L de BA y 1.7mg/L de $AgNO_3$. Combinaciones de BA, $AgNO_3$, GA_3 y ANA fueron ensayadas para el enraizamiento de los brotes, Sin embargo no fueron efectivas. Para estudiar la estructura de los callos por microscopia electrónica de rastreo, se realizaron tratamientos de fijación química y de criofijación. Los tratamientos de criofijación

incluyeron congelamiento rápido, congelamiento lento y enfriamiento lento. Aunque ninguno de los tratamientos preservó de manera efectiva los callos, se pudieron hacer algunas observaciones e inferencias relacionadas con la organización estructural de los callos.

To my parents, Ligia and Carlos, my sister, Giannina, and brother Kike, for giving me all the love and support during my whole life.

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Table of Content

Table List

Tables **Page**

Figure List

Figure **Page**

1. INTRODUCTION

1.1 Legumes

Legumes (Fabaceae family) are of special interest in agriculture world-wide because of their protein-rich seeds (grain legumes) and their use as animal food (forage legumes). They also fix gaseous nitrogen in symbiosis with *Rhizobium*, thus serving as organic nitrogen fertilizer, and therefore are important in developing sustainable agricultural practices and reducing the use of chemical fertilizers (Hammatt et al. 1986; Graham and Vance, 2003).

After cereals, legumes are the second crop in agricultural importance, representing about 13% of total land under cultivation in 2004. Specifically, grain legumes provide 33% of all dietary protein and nitrogen and 33% of processed vegetable oil for human consumption. At least 20 to 40% of the seed is protein (Gepts et al., 2005). Grain legumes are mainly cultivated in developing countries, where they accounted for 61.3 million hectares in 2002, compared to 8.5 million hectares in developed countries (Popelka et al., 2004).

Common beans (*Phaseolus vulgaris* L.) are important grain legumes and are considered a basic food that supplies not only proteins, but also calories,

vitamins and mineral salts in the human diet. Their cultivation extends over the five continents, with America and Africa as the main producers and consumers (Montilla 1996). Although beans are considered a low status food, they provide the second most important source of protein after maize (*Zea mays* L.), and the third most important source of calories after maize and cassava (*Manihot sculenta* Crantz) (David et al. 2003). As source of minerals, beans provide iron, phosphorus, magnesium, manganese, and to a lesser degree, zinc, copper and calcium. In many rural zones of Africa, where people have restricted economic means and micronutrient deficiencies are common, beans provide 10–20% of the adult requirement for a number of nutrients, mainly iron (Broughton et al. 2003).

1.2 The Genus *Phaseolus*

The genus *Phaseolus* (family Fabaceae, subfamily Papilionoideae, tribe Phaseoleae, subtribe Phaseolinae) includes several types of beans. Nevertheless, the word *bean* is used to designate two distinct botanical genera: *Phaseolus* and *Vigna,* the first one native to the Americas, and the second native to Africa (Milano, 2003).

There are about 50 species of *Phaseolus*, originated in Central and South America (Gepts, 2002), all of which are diploid, and most of which have 22 chromosomes (2n = 22; Gepts 2002). Of these, the most important domesticated species (in decreasing order of importance) are: common bean, lima bean (*P.*

lunatus L.), scarlet runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray), and year bean (*P. polyanthus* Greenman) - each with distinct adaptations and reproductive systems. The common bean can be easily adapted to *mesic* habitats (that is, it thrives in moderately moist habitats), while lima beans tolerate warm and humid habitats, tepary bean grows in hot and dry places, and scarlet and year beans prefer cool and humid environments.

The reproductive systems of common bean, lima bean and tepary bean are predominantly *cleistogamous* (flowers never fully open and are automatically self-fertilized); while year bean and runner bean are *outcrossing* (cross fertilization occurs between different individuals). Phylogenetically, lima bean is more distant from the other domesticated species, which are sibling species and constitute a *syngameon* (a unit of interbreeding in a hybridizing species group), although the degree of difficulty and the viability of crosses between them vary (Broughton et al. 2003).

1.2.1 Common bean (Phaseolus vulgaris L.)

The most economically and scientifically important species of the genus *Phaseolus*, the common bean, originated in Latin America where its wild progenitor (*P. vulgaris* var. mexicanus and var. aborigineus) has a wide distribution, ranging from northern Mexico to northwestern Argentina (Gepts, 2002). This is a polymorphic species, with stems that reach three to four meters

in length and roots with nitrogen nodules. Common bean is the second most important grain legume world-wide, only surpassed by soybean (*Glycine max* L. Merr). It complements cereals and other carbohydrate-rich foods, as it is a valuable source of calories, proteins, dietetic fiber, minerals and vitamins (Rodiño, 2000).

Common bean has been farmed in Latin America for many years. It is cultivated mainly for its green pods and fresh and dry grains, although in some countries of Latin America and Africa, the leaves and flowers are also consumed as fresh vegetables (David et al., 2003). Its production has been limited due to the susceptibility to pests and diseases; it is estimated that approximately 200 pathogens can affect this crop (Placencio and Mora-Nuñez, 2002).

1.3 Previous work in common bean

Even though it is unknown when this species became a crop of economic importance, plant breeding research in common bean as an organized activity began after 1930, and Mexico and Brazil were the first countries to implement plant breeding programs (Mora-Nuñez, 1997). In addition, for more than a century – from the studies of Mendel in the nineteenth century, who used this species to confirm results originally obtained from peas (*Pisum sp.*), to recent advances in the phylogeny of the *Phaseolus* genus using molecular techniques - *Phaseolus* species have been used in numerous research projects, particularly in

genetics and molecular genetics (Gepts, 2002). Through molecular studies, it has been confirmed that *P. vulgaris* belongs to a complex of species that includes *P. acutifolius, P. coccineus,* and *P. microcarpus,* all of which can be inter-crossed. For this reason, *P. vulgaris* has been used as a model system in studies that aim to elucidate the molecular basis of various processes that occur in agronomically important relatives (Broughton et al. 2003). Another feature that contributes to the attractiveness of common bean as an experimental crop species is its genome size, which is about 450 to 650 million base pairs (Mb)/haploid, having approximately single copies of all loci. Its genome size has been compared with that of rice, the smallest genome among crop species (McClean et al., 2004).

The limitations to crop improvement in common beans and their lack of genes for disease resistance, have favored the development of interspecific crosses between *Phaseolus* species. Regeneration of hybrids obtained through such crosses has only been achieved by embryo rescue (Broughton et al., 2003).

1.4 Plant Biotechnology

Biotechnology is the discipline that focuses on the exploitation of the properties of living organisms for the generation of valuable products. It is based on scientific and technical information from areas such as cell biology, morphology, biochemistry, physiology, molecular biology, genetics, and various technical fields (Borem et al., 2003).

In recent years, plant biotechnology has developed remarkable recombinant deoxyribonucleic acid (DNA) technologies and tissue culture techniques. Even though advances in DNA-technology have been of enormous consequences, it is tissue culture that allows the regeneration of plants with the desired characteristics introduced by DNA-techniques (Barz and Oksman, 2002).

1.4.1 In vitro culture

The in vitro culture of plants has been defined as the science that allows the growth of plant cells, tissues or isolated organs of the mother plant (known as *explants*) under controlled artificial conditions (George, 1993). In order for tissue culture to have applications in agriculture, its final goal must be the regeneration of whole plants (Mroginski and Roca, 1993). The objectives of *in vitro* culture can be numerous; but some of the possible applications are the following:

- Basic studies of physiology, genetics and biochemistry.
- Bioconversion and production of useful compounds.
- Generation and propagation pathogen-free plants.
- Conservation and exchange of germplasm

1.4.1.1 The culture environment

Plant cultures are initiated by placing one or more explants into a pre-sterilized container with a sterile nutrient medium that must satisfy both the chemical and physical requirements of the plant cells. These requirements have

to be met by the culture vessel, the growth medium, and the external environment (light, temperature, etc.). The growth medium should supply all the essential mineral ions required for growth and development and, since in many cases the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant, the culture medium must also include additional organic supplements, such as amino acids and vitamins. Additional physical factors, such as temperature, pH, gaseous environment, light (quality and duration) and osmotic pressure have to be maintained within acceptable limits (Slater et al. 2003).

A culture medium usually consists of a solution of salts supplying major and minor micronutrients for the growth of whole plants, along with vitamins, amino acids, an iron source, and a carbon-based energy source (usually sucrose) (George 1993). The most widely used medium was established by Murashige and Skoog in 1962, and its composition is given in Table 1.1.

This culture medium can be used in either liquid or solid forms, depending on the type of culture. For cultures of plant cells or tissues that must be grown on the surface of the medium, the medium is usually solidified ('*gelled*') using agar, a carbohydrate obtained from some species of red algae (seaweed) (Slater et al., 2003).

Table 1.1 Composition of MS culture medium, established by Murashige and Skoog (1962)

Essential element	Concentration in medium (mg/L)
Macroelements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Microelements	
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron source	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplement	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Glycine	2
Carbon sourced	
Sucrose	30000

1.4.1.2 Plant growth regulators

Plant growth regulators are compounds that have a regulatory, rather than a nutritional role in growth and development of plant tissues. These substances occur naturally, but some synthetic chemicals can have similar physiological activities (Slater, 2003). Effective concentrations of each regulator will vary and

will need to be adjusted according to the genotype of the plant to be cultured, the type of tissue or organ, and the nature and the stage of the culture. Some authors consider substances as plant growth regulators whenever they have been added exogenously (George, 1993).

There are several recognized classes of plant growth regulators, including:

- Auxins
- Cytokinins
- Gibberellins
- Ethylene
- Abscisic acid

Auxins.

Auxins are incorporated in media to promote growth of callus, cell suspensions, and organs and to regulate morphogenesis, especially in conjunction with cytokinins. Applied auxins seem to be capable of erasing the genetically programmed physiology of plant tissues, which had previously determined their differentiated state. The auxin *indole 3-acetic acid* (IAA) has also been used with other regulators to induce direct organogenesis, including rooting of microcuttings and meristem and shoot cultures. The use of IAA in plant cell culture media is limited because of its instability to both heat and light. It is more common to use stable chemical analogues of IAA as a source of auxin in plant cell culture, such as *2,4-Dichlorophenoxyacetic acid* (2,4-D), the most

commonly used auxin, which is extremely effective in most circumstances. Other auxins used in tissue culture are *3-indolebutyric acid* (IBA), and *1-naphthalene acetic acid* (NAA) (George 1993).

Cytokinins

The first cytokinin to be discovered was *6-furfurylaminopurine* (Kinetin). Cytokinins comprise a separate class of growth regulators whose effect is most noticeable in the stimulation of cell division, induction of adventitious shoot formation, overcoming of apical dominance, and release of buds from dormancy. This last effect is opposite to that of endogenous auxins, which cause apical dominance. It has also been found that cytokinins delay leaf senescence, induce stomatal opening and promote chloroplast development.

Some 25 natural cytokinins structurally related to kinetin have been identified. Two of these compounds used in plant tissue culture are *zeatin* (*4-hydroxy-3-methyl-trans-2-butenylaminopurine*) and *2-iP* (*N^6-(2–isopentenyl) adenine*) also called IPA. Nevertheless, they are not employed routinely due to their high costs. Rather, synthetic cytokinins, such as kinetin and BA (*6-benzylaminopurine*) are commonly used in micropropagation (George,1993).

Gibberellins

Gibberellins have been isolated from fungi and higher plants. The most important gibberellin in plants is probably *1-Gibberellic acid* (GA_1), but the most

widely available compound is *3-Gibberellic acid* (GA_3), which is a fungal product (Davies 1988). These compounds can influence growth and development of plants by increasing stem length, promoting flowering and inducing fruit set. The effect of gibberellins in plant tissue culture is diverse, and while in some cases they can inhibit the formation of shoots, roots, and embryos, in other cases they can increase the number of organs formed (George, 1993).

Ethylene

Ethylene is a gaseous, naturally occurring, plant growth regulator synthesized by methionine in many tissues, in response to stress. It is involved in fruit ripening, senescence and the abscission of leaves, and its use in plant tissue culture is not widespread (Davies, 1988). Ethylene is produced during the culture of all kinds of plant cells, tissues and organs. The rates of biosynthesis are increased if cells are subjected to any stress. Ethylene production is also greater during the senescence of cultured plant material, reaching high concentrations when suspension cultures start the stationary phase, the time at which nutrients are limiting and most of the cells are senescing (George, 1993).

In cell and callus culture, auxins generally increase the production of ethylene, and in some cases it has been suggested that the responses of plants to auxins are ultimately brought about by the ethylene produced in response to an auxin treatment. When ethylene builds up sufficiently, it can inhibit the growth and development of the plant cell cultures (Slater et al., 2003). It has been

reported that ethylene can prevent both organogenesis and somatic embryogenesis from callus culture; therefore, the addition of inhibitors to ethylene action, such as silver nitrate ($AgNO_3$), can stimulate the induction of organogenic and embryogenic process (George, 1993).

Abscicic acid

Abscisic acid (ABA) controls bud and seed dormancy, stomatal closure, leaf abscission, senescence and can inhibit the auxin-promoted cell wall acidification and loosening, which permit cell elongation. In tissue cultures ABA is not widely used, but sometimes promotes morphogenesis and growth, and inhibits callus growth (George, 1993).

1.4.1.3 Types of Culture

Cultures can be initiated from different types of plant materials. On that basis, cultures are classified as either *organ* or *undifferentiated cells* culture. Organs that can be cultured include *determinate* organs (those with limited growth), such as leaves, flowers and fruits; and *indeterminate* organs (those with potentially unlimited growth) like meristems, shoots, zygotic embryos and roots. On the other hand, the *undifferentiated cells culture* may be of one of three types: *callus* (an amorphous structure formed by actively dividing undifferentiated cells), *protoplast* (plant cell without cell wall) or *suspension cell* culture (population of plant cells dispersed in liquid medium) (George, 1993). Generally, younger, more

rapidly growing tissue (or tissue at an early stage of development) is most effective when used as culture material (Slater et al., 2003).

1.4.1.4 Plant Regeneration

Plant regeneration is the ability of protoplasts, single cells or tissues to develop into complete plants (Endress, 1994). Two techniques of tissue culture have been developed for the regeneration of whole plants: *somatic embryogenesis* and *organogenesis. Somatic embryogenesis* is the origin of embryos from one or more cells, without the mediation of sexual cells. On the other hand, *organogenesis* is defined as the capacity of the explants to develop *organs*, specifically buds and roots. The main difference between these two processes is that the product of embryogenesis is autonomous and is not connected to any other structure via vessel members (xylem); while through organogenesis, non-autonomous organs are formed on the explant. It has been reported that embryogenic and organogenic processes can be induced from specific layers of tissue, or from specific cells in the same explant (Jiménez, 2001).

Both organogenesis and embryogenesis can be induced in two ways: *directly*, - that is, from differentiated cells without proliferation of non-differentiated tissue; or *indirectly* - from cells of callus or suspension cultures that do not present organization or specialization (George, 1996). The regeneration of whole

organisms depends upon the concept of *totipotency*, which is a genetic latent capacity to produce a whole plant. In this sense, plant cell culture and regeneration provide the most compelling evidence for totipotency. In practical terms, identifying the culture conditions and stimuli required to manifest this totipotency can be extremely difficult, and it is still a largely empirical process (Slater, 2003).

1.4.1.5 Somatic Embryogenesis

Somatic embryogenesis involves the induction of embryo development through an ordered series of embryological stages without the fusion of gametes. This technique has been widely used to asexually propagate plant species of economical, ornamental, ecological or agronomic importance (George, 1996), and it has helped to elucidate the sequence of processes related to the formation and development of zygotic (sexual) embryos (Dodeman et al. 1997).

Somatic embryos are similar to zygotic embryos, since they are bipolar structures, having a shoot and a root axis, lacking vascular connections with the parental tissue (Mroginski and Roca, 1993), and capable of producing plantlets (George, 1993). Like zygotic embryos, somatic embryos go through a series of stages before they are able to regenerate plants. These are the pro-embryonic, globular, heart, torpedo and cotyledonary stages (Figure 1.1).

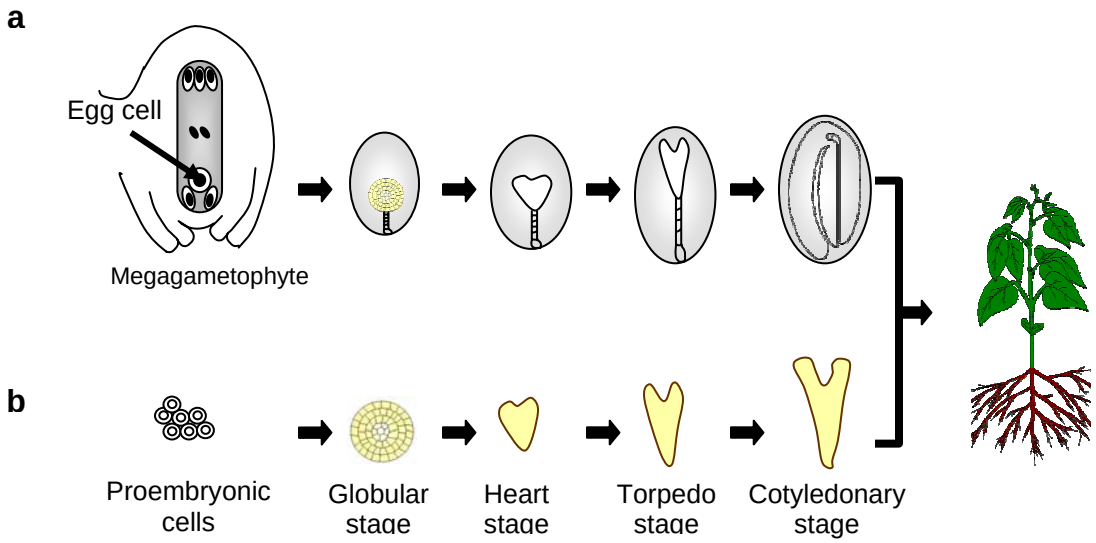

Figure 1.1 Schematic overview of stages of embryogenesis. a. Zygotic embryo development. b. somatic embryo development. (Adapted from Dodeman et al. 1997 and Arnold et al. 2002)

According to George (1993), under in vitro conditions *direct* somatic embryogenesis can only occur from cells of explants that are predetermined to develop embryos before their transfer to culture conditions. In contrast, *indirect* somatic embryogenesis requires that differentiated cells from an explant be induced to divide as callus, and then some of these cells become re-determined to form embryos. This induction requires that the explants be cultivated in an optimal medium supplemented with auxin, a growth regulator that stimulates cellular division. Figure 1.2 illustrates the process of somatic embryogenesis.

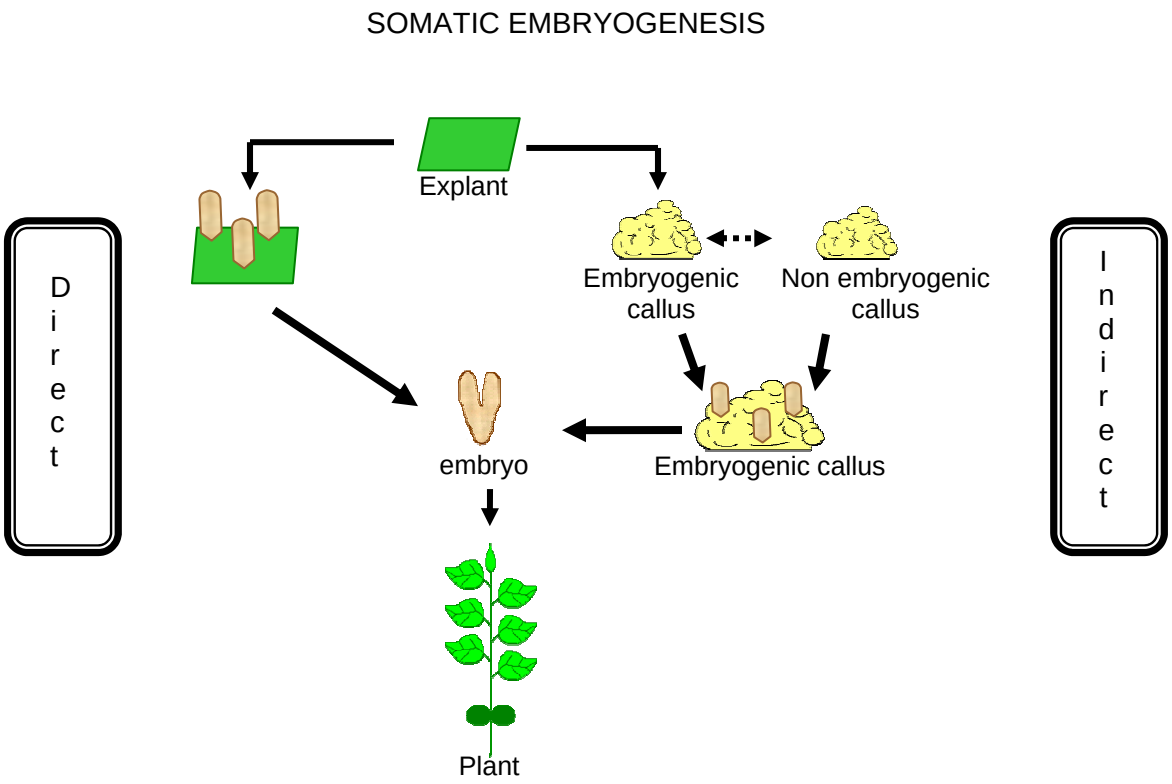

Figure 1.2 Somatic embryogenesis model. Adapted from George (1997)

Somatic embryogenesis can also occur naturally and it is known as *apomixis*. This phenomenon has been described in more than 400 species of plants belonging to 35 families, among which the Poaceae, Asteraceae, Rosaceae and Rutaceae are notable. Embryos arise directly from a somatic cell of the nucellus or from the integuments of the ovule (Ortiz et al. 2004).

1.4.1.6 Organogenesis

Organogenesis is concerned with the production of organs, either directly from an explant or from a callus culture. *Adventitious* organs (formed in unusual points of origin) can arise either from a callus culture or directly from an explant. Alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture. Figure 1.3 illustrates these processes.

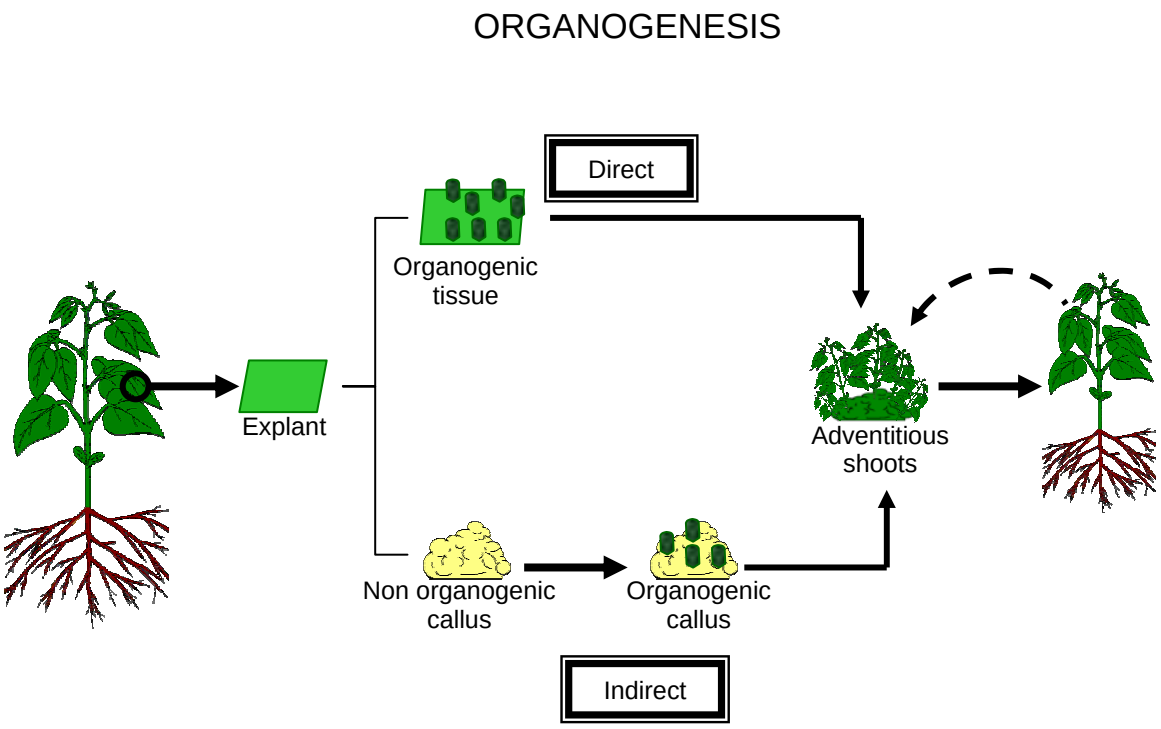

Figure 1.3 Organogenesis model, showing direct and indirect processes.

Typically, organogenesis is preceded by the appearance of small precursor cell populations or *meristemoids*. These structures can rise directly from certain cells in the primary explant or from the callus. Subsequently, they transform

gradually into shoot, root or floral apical meristems. (Hicks, 1994). This process must be preceded by changes in cellular organization such as cellular differentiation and interaction and reaction to specific signals (Litz and Jarret, 1993)

A diversity of methods of plant regeneration is now available. Some plant species may be amenable to regeneration by a variety of protocols; but some may only be regenerated by one method. Likewise, not all plant tissues are suited to every plant transformation method. There is, therefore, a need to find both a suitable plant tissue culture/regeneration regime, and a compatible plant transformation methodology (Slater, 2003).

1.4.2 In vitro culture of legumes

In vitro culture is a useful tool to facilitate the induction of genetic variability and the production of improved hybrids with tolerance to pathogens and some edaphic conditions. However, most of the grain legumes, including beans, remain recalcitrant when it comes to their proliferation through these techniques (Chandra and Pental, 2003). Only the forage legumes have been consistently propagated successfully by means of embryogenesis and organogenesis.

Soybean has been the most widely studied legume in tissue culture. Research has focused on the induction of embryogenic callus from cotyledonary

and hypocotyl explants, using different concentrations of dichlorophenoxyacetic acid (2,4-D). Kim et al. (2001) obtained embryogenic callus from edges of cotyledons cultured in MS medium containing 40 mg/L of 2,4-D, while the production of embryogenic clumps was obtained in MS suspension medium with 5 mg/L of 2,4-D and 0.5 mg/L of asparagine. This last protocol induced a 96% of regeneration. Similarly, Moon and Hildebrand (2003) induced and proliferated embryos with 40 and 5 mg/L of 2,4-D in solid medium containing MS salts and B5 vitamins; while liquid medium without plant regulators, as well as solid medium supplemented with 6% maltose and 0.5% activated charcoal, permitted the maturation of these embryos. In this report, the regeneration rate was relatively low; the highest rate was obtained when embryos were air-dried, and even then it only reached 35%.

2,4-D has not been the only auxin used for the production of somatic embryos in soybeans. For example, Bonacin et al. (2000) induced and proliferated embryos from immature cotyledons maintained under different concentrations of NAA, pHs and light intensities. They reported that 10mg/L of NAA at pH of 7.0 promoted the best production of embryos, while there were no significant differences under different light intensities. The auxin IBA has also been used, along with abscisic acid (ABA), to promote maturation of somatic embryos (Shoemaker et al.,1991).

The type of explant used as starting material for the production of callus may be as important as the type of growth regulator applied and its concentration. Coelho Da Silva et al. (2003), using cultures of hypocotyls and cotyledons maintained in darkness at 28°C, found that cotyledons produced the highest amount of callus when cultured with 1.0mg/L of 2,4-D and 0.1mg/L of Kinetin, while hypocotyls required twice the amount of 2,4-D (along with Kinetin at 0.1mg/L).

There is evidence suggesting that the genotype of soybean tested influences the embryogenic response. In 2002, Fernando et al. induced similar rates of embryogenic callus from two different soybean varieties, when concentrations of 40 mg/L of 2,4-D in one instance, and 0.5 mg/L in the other, were applied. For both varieties non-synchronous somatic embryogenesis, as well as some abnormal embryos (which presented only root meristem) were observed. Likewise, when Tomlin et al (1991) tested seventeen breeding lines of soybean, different responses where obtained for each line when concentrations of 136, 181 and 226 µM 2,4-D (30, 40 and 50mg/L, respectively) were applied.

Other legumes, such as *Medicago arborea*, alfalfa (*Medicago sativa*), and bird's-foot trefoil (*Lotus corniculatus*) have also been regenerated by means of somatic embryogenesis. In the first case, cotyledons and petioles of *Medicago arborea* were the best explants for the production of somatic embryos when they were cultured with 9µM 2,4-D (2mg/L) and 9µM Kinetin (2mg/L), and

subsequently transferred to kinetin-free MS medium with 2,4-D at 2.25µM (0.5mg/L) (Gallego et al. 2001). In alfalfa, callus induced from petioles and maintained in SH4K medium with 4.5µM 2,4-D (1mg/L) and 0.9µM Kinetin (0.2mg/L) consistently remained highly embryogenic, and the embryos induced showed vigorous germination (Tian et al. 2002). Akashi et al. (1998) established root cultures of *Lotus corniculatus* capable of producing somatic embryos without a phase of callus in hormone-free MS medium; the embryogenic capacity of callus was maintained when they were cultured in presence of 2.22 µM BA (10mg/L).

In relation to the propagation of *Phaseolus,* efforts have been made to establish *in vitro* cultures and to regenerate the plant by means of organogenesis and embryogenesis. In *Phaseolus coccineus*, for example, both organogenesis and somatic embryogenesis have been induced from callus derived from cotyledon explants. Specifically, embryogenesis was increased when glucose was used as a carbon source. TIBA (2,3,5-triiodobenzoic acid) and absicic acid (ABA) improved plant regeneration, while there were no differences in morphogenesis when zeatin (ZEA), DHZ (Dihidrozeatin) or N6-2-isopentyladenine (2iP) were added (Genga and Allavena, 1991).

Shoot regeneration through direct organogenesis has been achieved in *Phaseolus* species. For example, Mohamed et al. (1992a) induced multiple shoots and regeneration of fertile plants from embryogenic axes in common and

tepary beans. These results were achieved by culturing the axes in Gamborg's B5 medium supplemented with 5 or 10 µM BA (1.12 and 2.25 mg/L, respectively). Rooting was induced in medium without plant regulators. Similarly, Santalla et al. (1998) assayed a regeneration procedure for elite breeding lines of *P. vulgaris* and landraces of *P. coccineus* L. from seedlings' explants containing a cotyledon and a small portion of the split embryonic axis. The explants were cultured in MS medium supplemented with 15µM BA (3.4mg/L) and 2µM GA_3 (0.7mg/L). The morphology of buds and shoots developed was better in *P. coccineus* than in *P. vulgaris*, and 63% of the shoots in both species produced roots.

By means of indirect organogenesis, Dillen et al. (1996) obtained green callus and subsequent shoots of *P. acutifolius* after seven subcultures of callus from pedicel and bud explants in MS media supplemented with thidiazuron (TDZ) at 0.5mg/L and indole 3-acetic acid (IAA) at 0.25mg/L. Interestingly, each subculture lasted two months, so that adventitious shoots were obtained after approximately fourteen months of culture. Similar plant regulators and concentrations were used by Zambre et al. (2001) to induce organogenic callus from *P. polyanthus* Greenman, while regeneration of plants was achieved in 0.1mg/L of BA. 40% of the plants obtained with this protocol was established successfully in greenhouse conditions. This protocol was not effective to propagate *P. coccineus.* The researchers also grafted shoots of *P. polyanthus* on *P. vulgaris* seedling rootstock; but only 40% of these grafts resumed growth.

Efforts have been made to establish *in vitro* cultures and regenerate plants of *P. vulgaris,* by means of somatic embryogenesis and organogenesis. In relation to somatic embryogenesis, various physical-chemical factors have been examined as potential morphogenic agents. For example, globular structures were developed from callus cultured in MS medium supplemented with Kinetin (0.5mg/L), 2,4-D (0.2mg/L) and casein hydrolisate (50mg/L); nevertheless, these somatic embryos failed to complete their development (Martins and Sondahl, 1984). Similar results were obtained by Saunders et al. (1987). They found that 10 or 30mg/L of 2,4-D induced the development of translucent globular structures, which they called *neomorphs*. These globular structures did not develop into somatic embryos when they were transferred to MS medium with or without 2,4-D; they continued self-replicating without apparent shoot or root apices. Similarly, Hoyos (1990) did not find differences in number and quality of somatic embryos produced in presence of 10 to 30 mg/L of 2,4-D and transferred, either to a similar medium, or to a medium with 3-indol butyric acid (IBA). He reported no differences in embryogenic callus maintained in light or darkness.

As in soybean, there is evidence suggesting that in *P.vulgaris* the genotype influences the embryogenic response. Comparing two genotypes of common bean, Dobrev et al (2001) showed that the genotype with lower content of endogenous IAA developed better quality of embryos. Their studies also

revealed that endogenous content of IAA increased when the higher concentrations of 2,4-D were used (2mg/L).

There are also reported studies involving the regeneration of *P. vulgaris* through both indirect and direct organogenesis. In 1993 Mohamed et al. induced the formation of callus in presence of IAA and TDZ, from which shoots developed, eventually regenerating whole plants in 1mg/L of BA. The highest number of regenerated plants per explant (7) was obtained in callus developed from petioles maintained in 1µM BA or ABA (0.225 and 0.26mg/L, respectively); whereas CPPU (N-(2-chloro-4-pyridyl)-N'-phenylurea) or fluoridine inhibited the shoot induction and elongation. These results showed that both BA and ABA stimulate indirect somatic organogenesis in *P. vulgaris* (Veltcheva and Svetleva, 2005).

Regarding direct organogenesis, 10µM BA (2.25mg/L) promoted the development of buds from meristem explants; whereas plant regeneration occurred at a frequency of 73% when the meristems were cultured in 1µM NAA (0.2mg/L) (Kartha et al. 1981). Likewise, McClean and Grafton (1989) achieved the induction of multiple shoot formation in cotyledonary node explants on MS medium supplemented with 5µM BA (1.125mg/L).

Mohamed et al (1992a) regenerated plants from adventitious buds developed in zygotic embryonic axis in presence of 5µM (1.125mg/L) of BA and then

transferred to a medium without growth regulators. These plants had a survival rate of 100%. Furthermore, the same researchers, starting from cotyledonary nodes without lateral buds, induced from two to five times higher numbers of shoots when the explants were cultured at concentrations of 0.25 or 1µM CPPU or TDZ, than when grown in 5µM BA (Mohamed et al. 1992b). Similar results were obtained by Malik and Saxena (1992), who found that concentrations of TDZ lower than those of BA (10 and 80 µM, respectively) are required to obtain optimal shoot formation from buds of *P. vulgaris*.

Direct organogenesis has also been achieved using thin cell layer cultures (TCL) with epicotyl explants. Proliferation and further development of buds were induced culturing these explants in MS medium supplemented with BA and $AgNO_3$ (Carvalho et al. 2000). Positive effects on the production and elongation of shoots by BA and $AgNO_3$ were also found in indirect organogenesis induced from cotyledon explants (Guidolin, 2003)

As an alternative to regenerate plants of *Phaseolus*, rescue of zygotic embryos of *P. polyanthus* and *P. vulgaris* at the early heart-shaped stage was reported by Geerts et al. (1999). They reached 50% as the highest rate of germination when they used Gamborg medium supplemented with ABA at 0.025mg/L. In addition, Geerts et al (2000) reported recovery of embryos *P. vulgaris* hybrids through the culture of immature pods. In this instance, after

eight weeks of culture in Phillips medium with 0.1mg/L of naphtalenacetyc acid (NAA), the highest rate of germination was 45%.

In spite of the studies and the knowledge acquired, *Phaseolus vulgaris,* one of the most important grain legumes, remains recalcitrant to its propagation by tissue culture through somatic embryogenesis. This has hindered advances in the transformation and regeneration of plants showing tolerance to adverse environmental conditions. Research regarding the establishment of optimal protocols for the production and development embryos capable of developing into normal plants must, therefore, continue. This is the aim of this thesis: to establish protocols to induce indirect somatic embryogenesis and organogenesis that would in turn permit the regeneration of normal plants of common bean.

In the following chapters, the experimental procedure is explained, followed by the results, where description of the observations and data are presented. Then, the chapters of discussion and conclusions contrast the results obtained in this thesis with those obtained in previous reported investigations. Finally, recommendations are presented that may serve as guides for further experiments.

2. JUSTIFICATION

The common bean is an annual and autogamous legume with an important role in human nutrition and in agriculture (Carvalho et al., 2000). This species is the second most important source of protein after maize (*Zea mays*) and it is the third most important source of calories after cassava (David et al., 2003).

Phaseolus vulgaris is very susceptible to drought and to disease, which reduces its productivity significantly (Carvalho et al., 2000). The development of improved varieties is the main strategy to overcome such susceptibility, as well as to improve the nutritional quality and economic stability for farmers and costumers (David et al., 2003).

The regeneration of many legumes has been successfully obtained by means of somatic embryogenesis and organogenesis and has been reported by numerous researchers. However, the regeneration of common bean by somatic embryogenesis has been unsuccessful because of the recalcitrance of this species and the inability to advance the development of embryos past the globular stages. Similarly, organogenesis has been successful in very few occasions (Carvalho et al., 2000; and Guidolin, 2003). This limits the possibilities of genetic transformation of common bean. In order to overcome this difficulty, it is necessary to analyze and determine the chemical conditions and the type of

explant that allow a reliable regeneration of plants by both, somatic embryogenesis and organogenesis.

3. EXPERIMENTAL PROCEDURE

3.1 Culture conditions

Explants were cultured using a basal medium composed of MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg, 1968). This medium has been used by other researchers in somatic embryogenesis of soybean (Fernando et al. 2002; Tomlin et al. 2002; Moon and Hidelbrand, 2003) and in regeneration of common beans (Carvalho et al. 2000; Guidolin 2003). Agar (8g/L) was used as a gelling agent when semi-solid medium was needed. To induce callogenesis and embryogenesis, 10g/L of sucrose were added as a carbon and energy source; while to induce organogenesis, 30g/L of sucrose were added to the media (Table 3.1).

Aliquots of 10ml of basal medium were dispensed into 150 x 25mm culture tubes with 100ml capacity, which were covered with plastic caps. All instruments and culture media were autoclaved at 15psi and 121°C, for 20 minutes. After adding the explants, the tubes were sealed with parafilm. All the procedures to initially establish the in vitro cultures, as well as all subsequent transfers, were made in aseptic conditions using a laminar flow hood. The cultures were incubated using a photoperiod of 16 hours of light and 8hrs of darkness, referred to as the *standard photoperiod* from hereon.

Table 3.1 Composition of basal medium for callogenesis/embryogenesis and organogenesis in *P. vulgaris*

Essential element	Concentration in medium (mg/L)	
	Callogenesis/ embryogenesis	Organogenesis
Macroelements		
NH_4NO_3	1650	1650
KNO_3	1900	1900
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
Microelements		
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Iron source		
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3
Organic supplements		
Nicotinic acid	1	1
Pyridoxine-HCl	1	1
Thiamine-HCl	10	10
Carbon source		
Sucrose	10000	30000

3.2 Disinfection and germination of seeds

Seeds of 17 F3 lines of interspecific crosses between compatible lines of *Phaseolus vulgaris* and *P. coccineus*, were used to produce the initial explants. The parental lines were a *P. vulgaris* Florida dry bean breeding line 5-593 (FLA)

developed by M. Bassett, and a *P. coccineus* G35172, developed by CIAT. The resulting F1 plants were self pollinated twice to obtain the F3 seeds used in this work.

The largest seeds of each interspecific hybrid line were rinsed with distilled sterilized water, immersed in 95% ethanol for one minute, followed by immersion in a 20% (v/v) solution of commercial bleach (Clorox®) for 20 minutes, and finally rinsed three times with distilled sterilized water. To germinate seeds, they were placed horizontally on a solid MS medium diluted to half strength, without growth regulators.

During this stage, the percentage of germinated seedlings, as well as their appearance, was evaluated.

3.3 Selection of Type of Explant and Possible Callogenic and Embryogenic Lines from 17 F3 Interspecific Bean Hybrid Lines

Interspecific hybrid *Phaseolus vulgaris* x *P. coccineus* lines were used in order to test if interspecific hybridization resulted in an improved embryogenic response of *P. vulgaris,* since somatic embryogenesis can be induced in *P. coccineus* (Genga and Allavena, 1991), and not in *P. vulgaris*.

3.3.1 Induction of Callogenesis with 10mg/L of 2,4-D and Embryogenesis with 2,4-D and BA

Fifteen-day old seedlings from 17 hybrid lines were dissected into the following five types of explants: apical and basal cotyledonary portions, epicotyl, hypocotyl and radicle (Figure 3.1). To induce callus, these explants were cultured for 30 days on basal medium supplemented with 10mg/L of 2,4-D (Saunders et al. 1987; Hoyos 1990). The calli obtained were transferred to medium with 0.1mg/L of 2,4-D and 1mg/L of BA at 25°C, since Martins and Sondahl (1984) have suggested that the concentration of 2,4-D should be reduced in order to promote the development of embryos. Characteristics of callus growth, such as necrosis, color and type of callus (friable, nodular or compact) were evaluated before each transfer (every 30 days). Four units for each type of explant per F3 line were used.

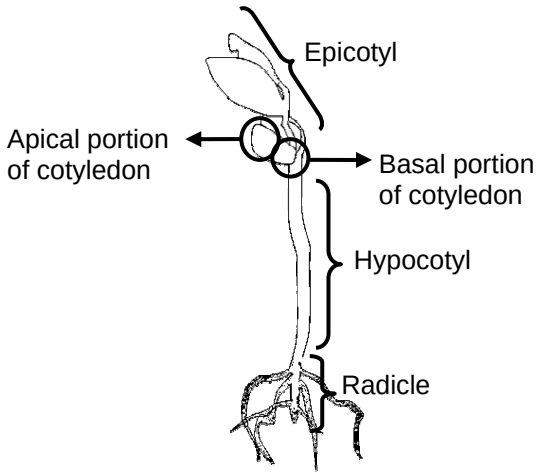

Figure 3.1 Explants obtained form fifteen day old seedling of P. vulgaris

3.3.2 Induction of callogenesis with 1mg/L of 2,4-D and embryogenesis with 2,4-D (0.1mg/L), BA (1mg/L) and Maltose (60g/L)

Explants were obtained from fifteen day-old plants and cultured for 30 days on a basal medium supplemented with 1mg/L of 2,4-D (Saunders et al. 1987; Mohamed et al. 1992). In order to induce embryogenesis, callus obtained from each explant was cultured on a basal MS medium supplemented with 0.1mg/L of 2,4-D and 1mg/L of benzilaminopurine (BA). The cultures were maintained under standard photoperiod conditions at 25°C for 20 days, and subsequently transferred to basal medium diluted to half of full strength , supplemented with maltose (60g/L) and BA (1mg/L), as suggested by Moon and Hidelbrand (2003) for soybean cultures.

After each transfer, callus formation, embryogenic capacity and qualitative traits of calli were evaluated for each explant. Two F3 lines of the 17 assayed were selected for their superior callogenic response. Four units for each type of explant per F3 line were used.

3.4 Callogenic and Embryogenic Response of the Two Selected Hybrid Lines

3.4.1 Callogenic Effect of 2,4-D in presence of 1mg/L of BA

Fresh explants of the two previously selected F3 lines were placed horizontally on a solid basal medium supplemented with 1mg/L of BA. Concentrations of 0, 0.01, 0.05 and 0.1mg/L of 2,4-D were added as described in Table 3.2. The treatments were incubated under standard photoperiod at 25°C. After 30 days in culture, the number of explants developing into callus and the morphological characteristics of both, the explant and callus, were evaluated. Each treatment consisted of eight explants.

Table 3.2 Treatments for induction of callogenesis in two hybrid lines of P. vulgaris and P. coccineus, using different concentrations of 2,4-D in presence of BA

BA (mg/l)	2,4-D (mg/l)	Explant
1	0	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
	0.01	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
	0.05	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
	0.1	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl

3.4.2 Callogenic Effect of 2,4-D at 29°C

Explants from seedlings were cultured on solid basal medium supplemented with 0, 0.01, 0.05 and 0.1mg/L of 2,4-D (Table 3.3). The treatments were maintained in darkness at 29°C. The number of explants developing into callus and the morphological characteristics of both, the explant and callus, were evaluated after 30 days of culture. Eight replicas of each type of explant were cultured for each treatment assayed.

Table 3. 3 Treatments for induction of callogenesis in two hybrid lines of *P. vulgaris* and *P. coccineus*, using different concentrations of 2,4-D at 29°C

2,4-D (mg/l)	Explant
0	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
0.01	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
0.05	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl
0.1	Apical portion of cotyledon Basal portion of cotyledon Epicotyl Radicle Hypocotyl

3.5 Maturation of Nodular Callus

Nodular calli obtained from hypocotyl and radical explants cultured in 0.1mg/L of 2,4-D and 1mg/L of BA were subcultured and multiplied under the same conditions. This calli were used to perform the experiments of maturation of callus described below.

3.5.1 The Effect of Air-drying

To essay the effect of desiccation on maturation of common bean nodular callus, an experiment was designed according to maturation experiments in soybean (Santarem and Finer 1999, Kim et al. 2001 and Moon and Hidelbrand 2003). Nodular calli were air-dried for 15 days in petri dishes lined with filter paper and sealed with parafilm. The calli were then transferred back to the medium from which they originated (that is, to one of the media presented in Table 3.2). Observations were made on the development and organization of the callus. In this trial 17 replicas of the experimental unit were used. Each experimental unit was represented by one callus cultured in a tube.

3.5.2 The Effect of Liquid Medium

In order to test the effect of liquid medium on the formation of embryos, an experiment was designed similar to the experiments of Martins ad Sondahl (1984), Saunders et al (1987), Hoyos (1990), and Mohamed et al. (1992).

Nodular calli were transferred from each of the media presented in Table 3.2, to liquid basal medium supplemented with 2,4-D (0.1mg/L) and BA (1.0 mg/L), and maintained under continuous agitation at 150rpm and 29°C (Table 3.4). Every 15 days the cultures were transferred to fresh medium, and after 30 days, data was taken on the characteristics of the nodular calli, including color, necrosis and proliferation. Each flask represented an experimental unit, and 20 replicas were used for this assay.

Table 3.4 Experiments for determination of effect of the type of medium

Type of medium	2,4-D (mg/L)	BA (mg/L)
Solid (Control)	0.1	1
Liquid	0.1	1

3.5.3 The Effect of $AgNO_3$

In order to evaluate the effect of silver nitrate ($AgNO_3$) on embryogenesis, nodular calli were transferred from a medium supplemented with 0.1mg/L of 2,4-D and 1mg/L of BA to one with similar composition, but supplemented with 1.7 mg/L of $AgNO_3$ (Table 3.5). Silver nitrate was successfully used in experiments to enhance the formation of somatic embryos in *Brassica sp (*Biddington et al. 1988), *maize* (Duncan and Widholm 1987 cited by George 1993), and shoots of common bean (Carvalho et al 2000) and cotton (Ouma et al. 2004). The

treatments were maintained under standard photoperiod at 25°C. Observations on the development of calli were made after 30 days of culture. 20 replicas were used in this experiment.

Table 3.5 Treatments with silver nitrate for the induction of embryogenic processes in two hybrid lines of P. vulgaris and P. coccineus

$AgNO_3$ (mg/L)	2,4-D (mg/L)	BA (mg/L)
0 (Control)	0.1	1
1.7	0.1	1

3.5.4 The Effect of Dessication in Combination with $AgNO_3$

Nodular calli were air-dried for 15 days, as explained in section 3.5.1, and transferred to a basal medium with 2,4-D and BA (0.1 and 1mg/L, respectively) with or without $AgNO_3$ (Table 3.6). Data on the development and organization of the calli were taken. Each Petri dish represented an experimental unit. A total of 24 experimental units were used.

Table 3. 6 Treatments to callus air-dried for 15 days, and transferred to medium with or without $AgNO_3$

Explant	$AgNO_3$ (mg/L)	2,4-D (mg/L)	BA (mg/L)
Air-dried	0 (Control)	0.1	1
	1.7	0.1	1

3.5.5 The Effect of Liquid Medium and $AgNO_3$

Nodular calli were transferred from a medium with 0.1mg/L of 2,4-D and 1mg/L of BA to either liquid or solid media with similar composition, supplemented with 1.7mg/L of $AgNO_3$ (Table 3.7). Data collected on the calli included color, type of callus and presence of necrosis in the explant. Ten replicas for each experimental unit were used.

Table 3.7 Treatments used to test the effect of liquid and solid medium in somatic embryogenesis in callus

Medium type	Regulators combination
Solid	2,4-D (0.1 mg/L) + BA (1 mg/L) + $AgNO_3$ (1.7 mg/L)
Liquid	2,4-D (0.1 mg/L) + BA (1 mg/L) + $AgNO_3$ (1.7 mg/L)

3.6 Organogenesis

3.6.1 Proliferation

Lateral and adventitious buds obtained from epicotyls were transferred to basal medium supplemented with 0.5mg/L of BA (McClean and Grafton 1989). After 30 days, buds were transferred to a medium as proposed by Carvalho et al. (2000) consisting of MS salts, B5 vitamins, 2.25mg/L BA and 1.7mg/L $AgNO_3$. Data of qualitative traits of buds such as necrosis, color and number of buds were taken after each transfer.

3.6.2 Elongation

Buds were transferred to basal medium supplemented with 2.25mg/L BA, 1.7 mg/L $AgNO_3$, and 1.5 mg/L GA_3 (Carvalho et al. 2000). Morphological traits of shoots, as well as their height, were evaluated.

3.6.3 Rooting

In order to promote rooting and based on Carvalho et al. (2000), who reported that NAA and $AgNO_3$ favor this process, shoots with 1cm of height were transferred to basal medium supplemented with BA, GA_3, NAA and $AgNO_3$ in

different combinations (Table 3.8). The height of shoots and the number and length of roots were evaluated after 30 days of culture.

Table 3.8 Treatments with BA, GA_3, NAA and $AgNO_3$ to promote rooting

Treatment	Concentration (mg/L)			
	BA	**GA_3**	**NAA**	**$AgNO_3$**
Control	2.25	1.5	0	1.7
1	0	0	0.2	1.7
2	2.25	1.5	0.2	1.7
3	2.25	1.5	0.4	1.7
4	4.5	1.5	0.2	1.7
5	2.25	1.5	0.2	0

3.7 Characterization of Calli from Maturation Treatments by Microscopy Techniques

Samples of the calli obtained in the different maturation treatments applied to F3 hybrid lines 133 and 155, were observed by both, stereomicroscopy and scanning electron microscopy (SEM).

The calli samples were collected and immediately observed in a Nikon SMZ 1500 stereoscope or in an Olympus SZ60 stereoscope. During the

observation, calli were dissected to reveal possible embryo-like structures within them, and digital images were obtained.

Additionally, characterization of the calli was performed by scanning electron microscopy (SEM) using a JEOL 5410LV. Standard steps of chemical fixation, dehydration, and coating were necessary to prepare the specimens for scanning electron microscopy. Samples were chemically fixed with 2% glutaraldehyde and 2.5% paraformaldehyde in a phosphate buffered saline (0.1M) solution for 24 hours, rinsed several times with buffer, and dehydrated by transferring them every 45 minutes to solutions with increasing ethanol concentrations (25, 35, 45, 55, 65, 75, 85, 95 and 100%). After three rinses with 100% ethanol, the samples were submitted to critical point drying and then sputter-coated with gold/palladium to finally be analyzed by SEM. Secondary electron images (SEI) were obtained using a voltage of 5KV, and images were captured digitally.

3.8 Scanning Electron Microscopy

In order to identify the most effective fixation protocol for calli of *P. vulgaris*, calli which had been cultured under similar conditions, were either fixed chemically or cryo-fixed. Chemical fixation was done as described in section 3.7, while cryofixation protocols included three options: rapid freezing, slow freezing

and slow controlled cooling. All calli used in these experiments came from a single suspension culture that was divided into two separate samples. One was supplemented with 10% (v/v) of the cryoprotectant dimethylsulfoxide (DMSO) (Martínez-Montero et al., 2000), and the other half was maintained without DMSO. These treatments are summarized in Table 3.9.

Table 3.9 Treatments to fixate calli samples of common bean pretreated or not with DMSO

Fixation	Pretreatment	Treatment
Chemical	-	Glutaraldehyde and paraformaldehyde
Cryofixation	Non-pretreated with DMSO	Rapid freezing by liquid propane Rapid freezing by liquid nitrogen Slow cooling Slow freezing
	Pretreated with DMSO	Rapid freezing by liquid propane Rapid freezing by liquid nitrogen Slow cooling Slow freezing

Rapid freezing was accomplished by immersion of the calli in either liquid propane (-180°C) or liquid nitrogen (-196°C), using a setup similar to that shown in Figure 3.2. Liquid nitrogen was used to cool down a hollowed aluminum cylinder, which was in turn used to liquefy gaseous propane. The calli were extracted from the liquid suspension using a micropipette with its tip broken off (to increase the diameter through which the calli would enter) and quickly dropped into the liquid propane or liquid nitrogen. Frozen samples were kept in liquid nitrogen to avoid thawing.

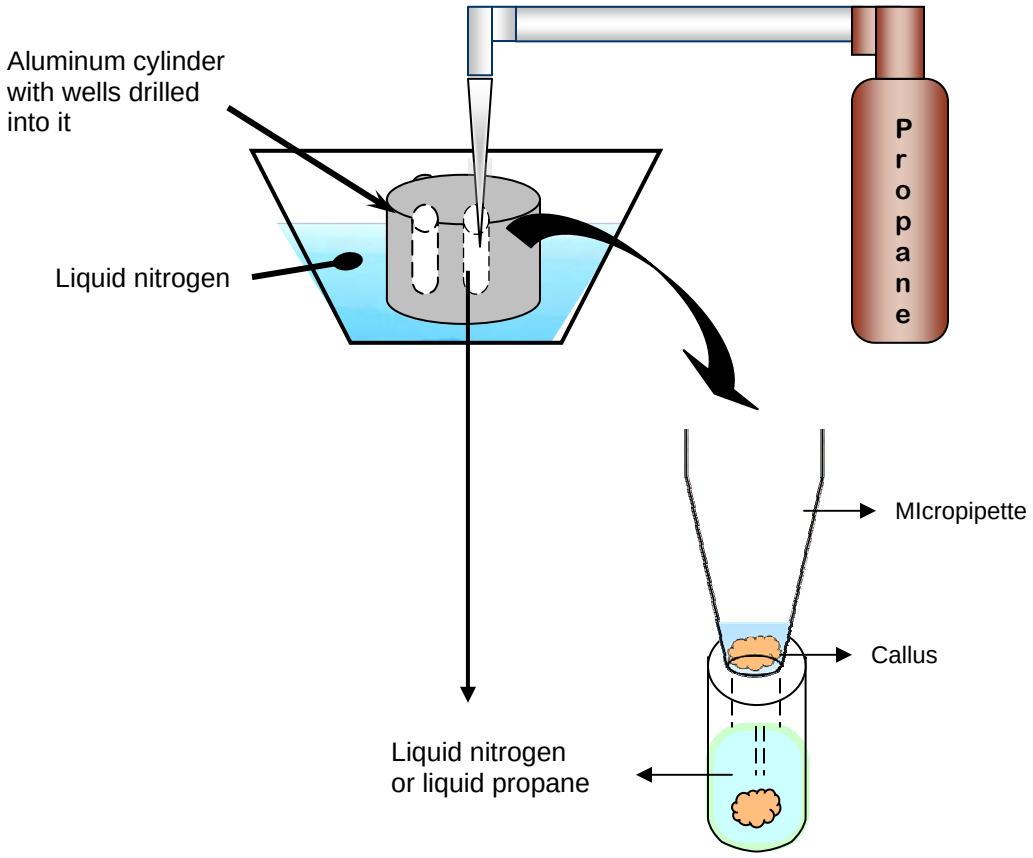

Figure 3. 2 System for rapid freezing with liquid propane or liquid nitrogen.

The slow freezing treatment was carried out by direct transfer of fresh calli to a freeze drying machine (Plus 6 LabConco®) for 48 hours. This machine drops the temperature of the samples to about -60ºC in approximately 3 minutes, and then sublimation of the ice occurs under vacuum (1µbar), rendering the sample dry and ready for sputter-coating and observation under the SEM.

Finally, slow cooling treatment was made as suggested by Martínez-Montero et al., (2000) by immersing calli in an ethanol bath at -80°C for 30 minutes, followed by immersion in liquid nitrogen for 2 hours.

Whether the calli were fixed by rapid freezing, slow freezing or slow cooling, they were eventually transferred to a freeze drying machine (Plus 6 LabConco®) for 48 hours at -60°C and 1µbar. Subsequently, the calli samples were mounted on aluminum stubs, sputter-coated with gold/palladium, and placed in a desiccator up until the time of examination by scanning electron microscopy. Two callus samples from each fixation protocol were examined in a JEOL 5410LV at 5KV.

Since preliminary observations of the rapid freezing and slow freezing assays suggested that these techniques are more successful than the other protocols in the preservation of morphological characteristics, these two assays were repeated and expanded. This time, ten callus samples were processed, and the freeze drying time was increased to 72 hours (at 1µbar at -60°C) to assure water sublimation.

In addition, three samples of fresh callus from the suspension culture were observed directly in the SEM using the low vacuum scanning mode. A pressure of 350µbar and 10KV to 15KV were used to observe the samples.

3.9 Experimental Design

The experiments were developed according to a complete randomized design, where each experimental unit was represented by an explant, or a seed during germination, or a portion of callus in other essays. The collected quantitative data was displayed as averages and percentages.

4. RESULTS

4.1 Disinfection and Germination of Seeds

After fifteen days of culture, 136 of 144 seeds (95%) had germinated and none of the cultures had become contaminated. The seedling reached an average of 3.5 cm of height with elongation of epicotyl, hypocotyl and roots. The first non-expanded leaves were apparent.

4.2 Selection of Type of Explant and Possible Callogenic and Embryogenic Lines from 17 F3 Interspecific Bean Hybrid Lines

4.2.1 Induction of Callogenesis with 10mg/L of 2,4-D and Embryogenesis with 2,4-D and BA

The callogenic response of apical and basal cotyledonary portions, epicotyl, hypocotyl and radical explants of the 17 hybrid lines cultured in basal medium supplemented with 10mg/L of 2,4-D presented significant differences ($p<0.005$. Appendix 1 and 2). For example, while there was no significant difference in the percentage of callus growth between explants of the basal part of cotyledons (93%), epicotyls (91%), hypocotyls (97%), and radicles (91%), a significantly lower percentage of explants from apical parts of the cotyledons (73%) developed calli.

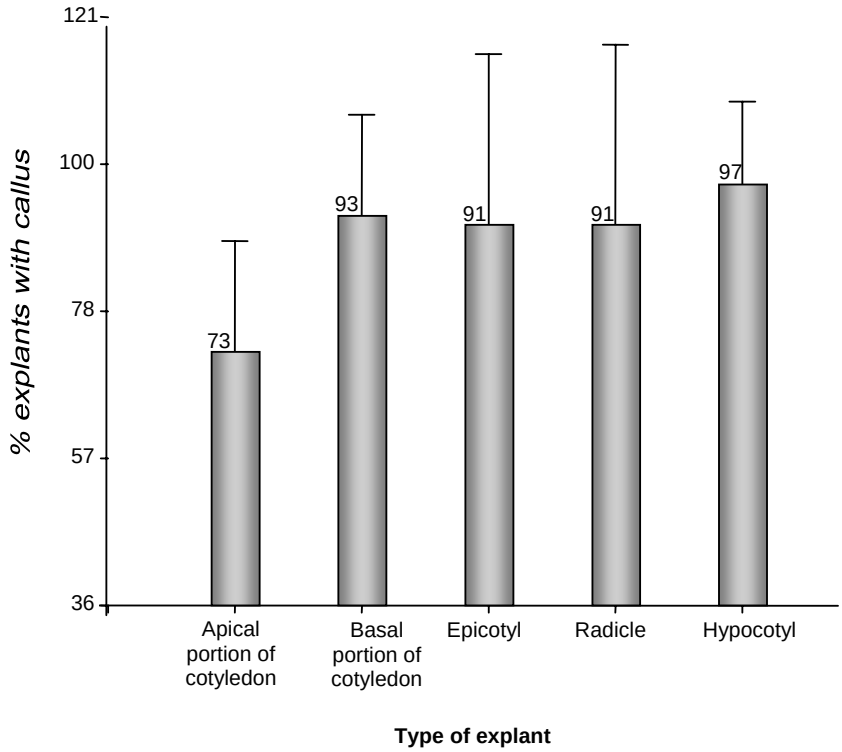

Figure 4.1 Callogenic response of cotyledon, epicotyl, hypocotyl and radical explants in presence of 10mg/L of 2,4-D. Each column is average of 17 hybrids (4 units per type of explant per hybrid). DMS=25.2%

In every case, callus was produced initially at the cut-zones within 10 to 15 days of culture, and in the case of epicotyl, radicle and hypocotyl explants, callus growth subsequently extended through most of their surfaces. On the other hand, callus eventually covered only about half of the surface of explants from apical and basal portions of cotyledons. All the explants, except radicles, developed only nodular callus; radicle explants produced both nodular and friable callus (Table 4.1). The color of the calli obtained ranged from brownish to yellowish and whitish.

Table 4.1 Callogenic response of explants from seedlings cultured in presence of 10mg/L of 2,4-D.

Explant type	Total explants	Callus		Explants with Necrosis (%)
		Type	Color	
Apical portion of cotyledon	68	nd	br, ye	48
Basal portion of cotyledon	68	nd	ye, gr, br	61
Epicotyl	68	nd	ye, wh, br	16
Radicle	68	fr, nd	ye, br, wh	4
Hypocotyl	68	nd	ye, gr, wh	15

fr = friable, nd = nodular, gr = greenish, ye = yellowish, br = brownish, wh = whitish

Necrosis also occurred to different extents in calli derived from different types of explants. The highest percentages of necrosis were observed in both apical and basal portions of cotyledons (48% and 61%, respectively), while radicles presented the lowest percentage (4%). Necrosis was observed initially at the cut-zone in every type of explant. Additionally on apical and basal portions of cotyledons, small necrotic zones were also observed dispersed on the surface of the cotyledon, which eventually invaded about one third of the entire explant, thus hindering the production of calli. On the other hand, necrosis observed in explants of epicotyl, radicle and hypocotyl did not inhibit callogenesis.

When the calli obtained from all types of explants cultured with 10mg/L of 2,4-D were transferred to a basal medium with 0.1mg/L 2,4-D and 1mg/L of BA to induce embryogenesis (Genga and Allavena, 1991), their response in terms of proliferation and appearance was similar in almost every case. After 30 days of

incubation, the calli from the different explants had a yellowish, greenish and brownish coloration with friable and nodular zones (Table 4.2). Callus originated from radicle and hypocotyl tissues developed globular structures, from which roots, in turn, developed. In one callus from a hypocotyl explant, a heart-like structure formed after 20 days of culture; but it spontaneously reversed into callus. Approximately 5% of epicotyl, radicle and hypocotyl explants did not produced calli.

Table 4.2 Response of nodular callus transfer to 0.1mg/L of 2,4-D and 1mg/L of BA

Explant type	Total explants	Callus		Explants with Necrosis (%)
		Type	Color	
Apical portion of cotyledon	37	fr, nd	gr, ye, br	60
Basal portion of cotyledon	59	fr, nd	ye,br,gr	68
Epicotyl	65	fr, nd	gr, ye,br	54
Radicle	65	fr,nd	ye, bg, wh, br	40
Hypocotyl	67	nd-fr,nd	gr, ye, br	77

fr = friable, nd = nodular, gr = greenish, ye = yellowish, br = brownish, wh = whitish

Necrosis was observed in callus from every type of explant, ranging from 40.6% in calli from radicle explants, up to 77.3% of calli from hypocotyls. In all cases, necrosis covered less than one third of each callus.

4.2.2 Induction of Callogenesis with 1mg/L of 2,4-D and Embryogenesis with 0.1mg/L of 2,4-D, 1mg/L of BA and 60g/L of Maltose

The callogenic response of explants cultured on basal medium with 1mg/L of 2,4-D was different according to the type of primary explant cultured ($p<0.05$. Appendix 3 and 4). Apical portions of cotyledons afforded the lower percentage (21.31%) of callus production (Figure 4.2) in contrast with 86%, for basal portions of cotyledons, and 100% for epicotyl, radicle and hypocotyl explants.

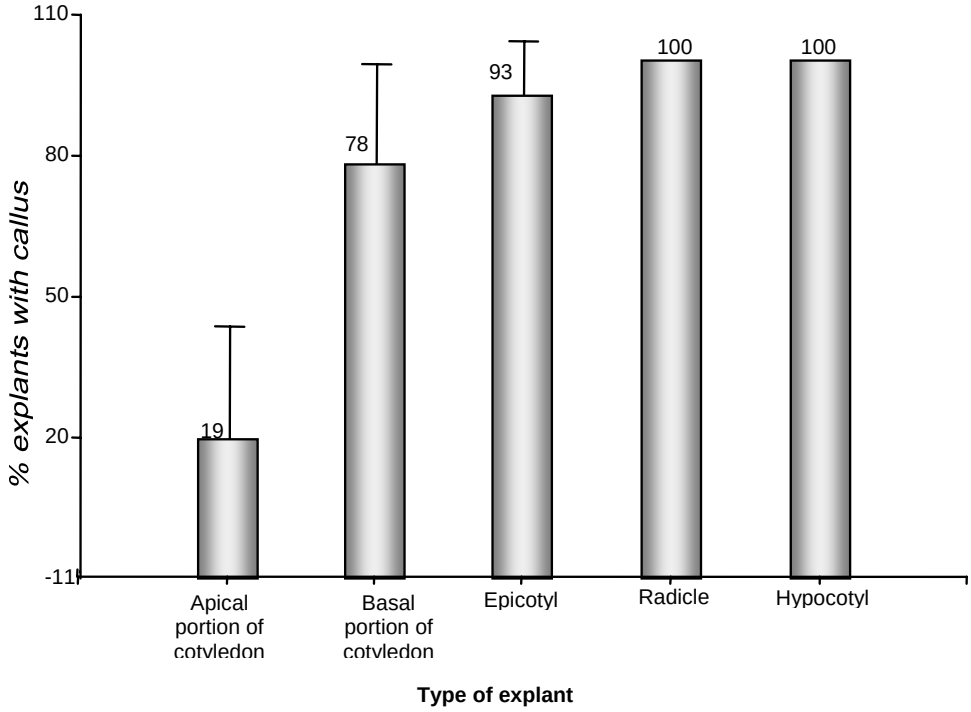

Figure 4.2 Callogenic response of cotyledon, epicotyl, hypocotyl and radicle explants in presence of 1mg/L 2,4-D. each column is average of 17 hybrids (4 explants per hybrid). DMS= 11.2%

All calli produced were entirely friable (Table 4.3). The apical and basal portions of cotyledons presented a blackish coloration at the cut zone within which small whitish and greenish calli often developed. The size of these calli was approximately one third of the size of the explants. Radicle and hypocotyl explants produced whitish calli that completely covered the explants; whereas, most of the calli produced from epicotyls covered about two thirds of the explants. In some of the epicotyl explants (about 28%), apical buds with clorotic leaves developed. Radicle and hypocotyl explants also produced roots (about 38%).

Table 4.3 Average response of cotyledons, epycotyl, hypocotyl and radicle explants after 30 days of culture in presence of 1mg/L of 2,4-D

Explant	Total explants	Callus		Explants with Necrosis (%)
		Type	Color	
Apical portion cotyledon	62	fr	wh-gr	88
Basal portion cotyledon	58	fr	wh-gr	63
Epicotyl	65	fr	wh	0
Radicle	68	fr	wh	0
Hypocotyl	68	fr	wh	0

fr = friable, nd = nodular, gr = green, ye = yellow, br = brown, wh = white

Necrosis of calli was evident in 88 and 63% of apical and basal portions of cotyledons and invaded about one third to one half of the explants' surface after 30 days of culture. Most explants became entirely necrotic when they were maintained in the same culture medium for two months. For the other types of explants (epicotyl, hypocotyl and radicle), no necrosis was observed.

The calli obtained in 1mg/L 2,4-D were transferred to basal medium with 0.1mg/L of 2,4-D and 1mg/L of BA. Calli from epicotyl, radicle and hypocotyl explants were friable, while calli from apical and basal portions of cotyledons exhibited both friable and nodular characteristics. Necrosis was evident in most of the calli from both apical and basal portions of cotyledons (aproximately 93%), covering half of each callus, and it was much lower in calli from epicotyl and radicle explants (16.1 and 6.3%, respectively), in which necrosis invaded less than a third of the explants' surface. Hypocotyl explants developed calli free of necrosis (Table 4.4).

Table 4.4 Response of calli from cotyledons, epycotyl, hypocotyl and radicle explants after 30 days of culture in presence of 0.1mg/L of 2,4-D and 1mg/L of BA

Explant	Total explants	Callus		Explants with Necrosis (%)
		Type	Color	
Apical portion cotyledon	61	fr, nd	gr, ye, br	93.4
Basal portion cotyledon	59	fr, nd	br, gr, wh	93.3
Epicotyl	65	Fr	ye, wh, gr, br	16.1
Radicle	68	Fr	ye, br, wh	6.3
Hypocotyl	68	Fr	br, ye, wh	0

fr = friable, nd = nodular, gr = green, ye = yellow, br = brown, wh = white

Calli grown in 0.1mg/L of 2,4-D and 1mg/L of BA were subcultured again in basal medium supplemented with maltose and BA to induce embryogenesis (Moon and Hidelbrand, 2003). In this case, most of the calli obtained from both apical and basal portions of cotyledons were nodular, while calli growing from the other types of explants were predominantly friable. Brownish, yellowish and

greenish colors were observed in calli produced from cotyledons and epicotyl explants; while those from radicle and hypocotyl explants, were largely brownish and yellowish (Table 4.5). There was no evidence of embryogenesis in any of the calli.

Table 4.5 Average response of calli from cotyledon, epicotyl, hypocotyl and radicle explants after 30 days of culture in presence of 60mg/L of maltose, and 1mg/L of BA.

Explant	Total explants	Callus		Explants with Necrosis (%)
		Type	Color	
Apical portion cotyledon	37	nd	br, ye, gr	66.7
Basal portion cotyledon	50	nd	br, ye, gr	70.0
Epicotyl	63	fr	br, ye, gr	50.0
Radicle	67	fr	ye, br	37.0
Hypocotyl	68	fr	ye, br	56.7

fr = friable, nd = nodular, gr = greenish, ye = yellowish, br = brownish, wh = whitish

The percentages of necrosis developing in calli coming from cotyledonary portions (both apical and basal) were again higher than the percentages from epicotyl, hypocotyl and radicle explants (Table 4.5).

As shown in Figure 4.3, all of the 17 F3 lines tested produced calli in presence of both concentrations of 2,4-D tested (1mg/L and 10mg/L). Nonetheless, the main criteria for the selection of lines were the proliferation of *nodular* calli and the lowest possible degree of necrosis (Figure 4.4) in both, explants and calli. These characteristics were best observed in lines 133 and 155, which were, therefore, selected for the remaining experiments to induce callogenesis and embryogenesis.

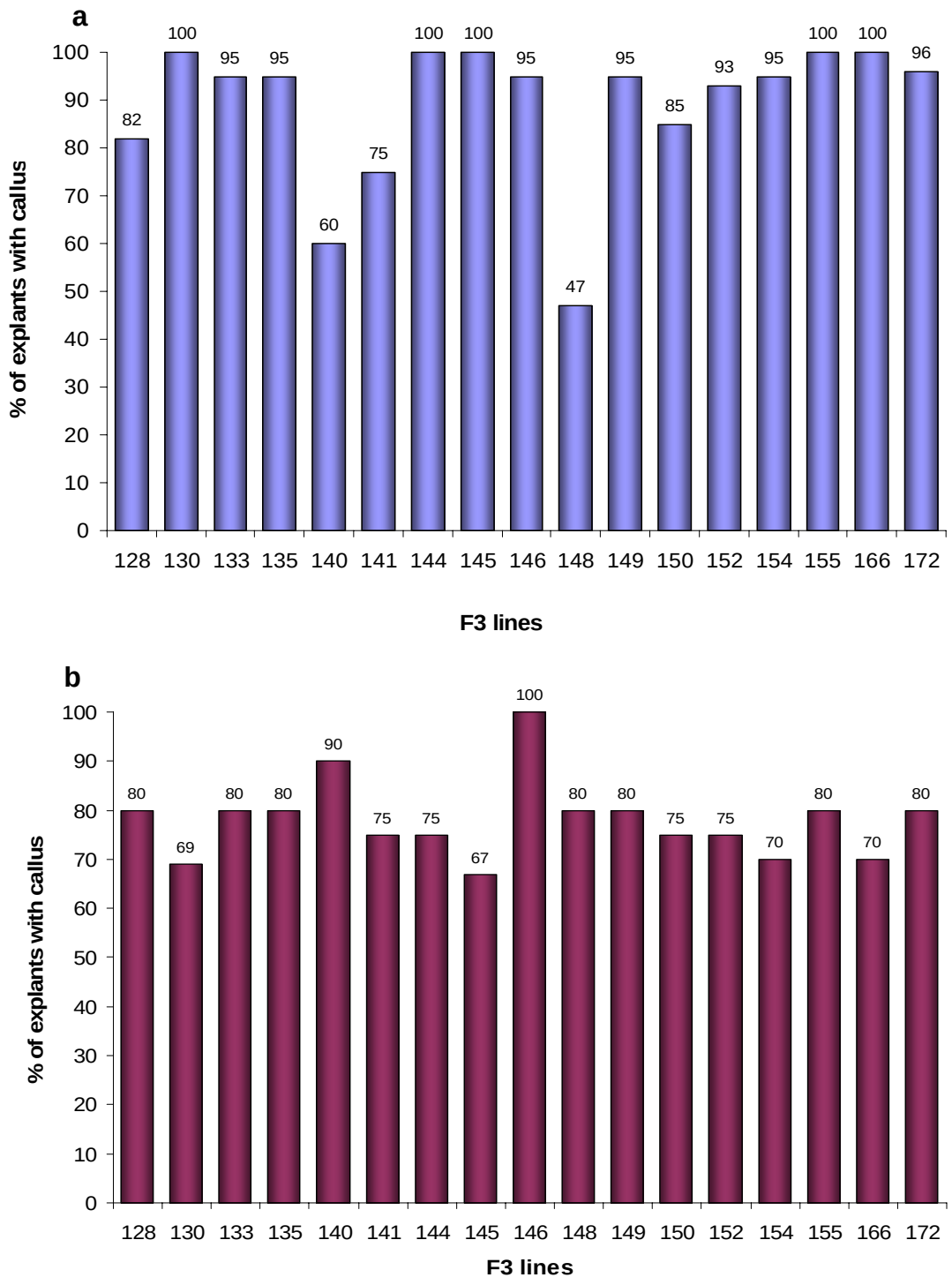

Figure 4.3 Percentages of explants of 17 hybrid lines that developed calli. a) In presence of 10mg/L of ,4-D. b) In presence of 1mg/L of 2,4-D

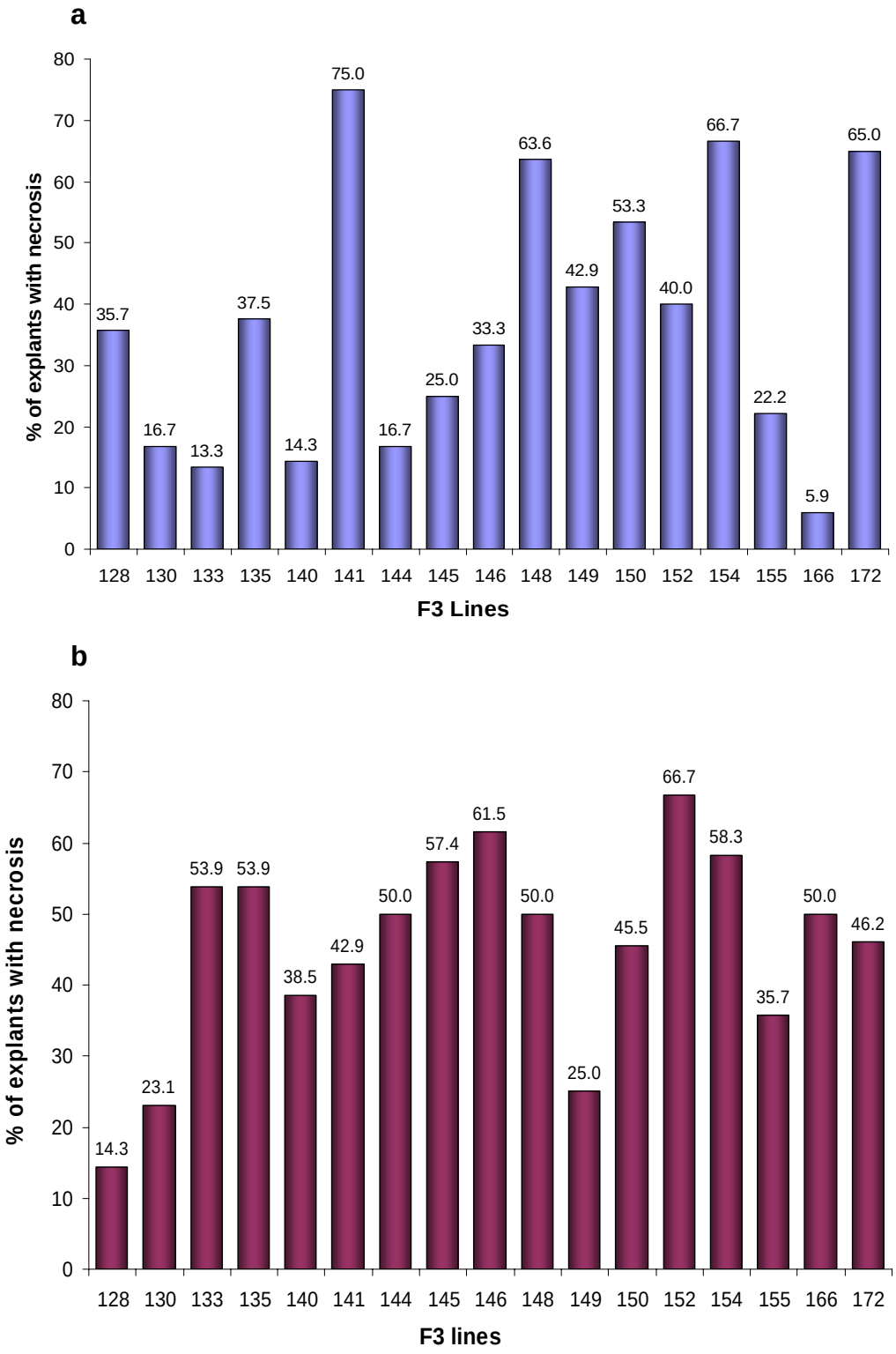

Figure 4.4 Percentages of explants of 17 hybrid lines that presented necrosis. a) In presence of 10mg/L of ,4-D. b) In presence of 1mg/L of 2,4-D

Based on our previous results (production and proliferation of nodular calli and formation of globular structures), a lower concentration of auxin (0.1mg/L) was used in all subsequent experiments aimed at elucidating a protocol for the induction on both callogenic and embryogenic processes.

4.3 Callogenic and Embryogenic Response of the 133 and 155 F_3 Hybrid Lines

The results obtained in this section and the following were similar in both hybrids selected (133 and 155). For that reason, the following description of the results does not make a distinction between the two hybrid lines.

4.3.1 Callogenic effect of 2,4-D in presence of 1mg/L BA

After 30 days of culture under different concentrations of 2,4-D (0, 0.01, 0.05 and 0.1mg/L), and 1mg/L of BA, the results shown in Table 4.6 were obtained. All of the radicle and hypocotyl explants produced callus. The highest percentages of explants that developed callus were observed in treatments with 0.05 and 0.1mg/L of 2,4-D (87.5% and 100%, respectively). The lowest percentage of callus development was observed for all treatments applied to apical and basal portions of cotyledons, with only one exception (62.5% of basal portions of cotyledons cultured in the presence of 0.05mg/L of 2,4-D formed calli) (Table 4.6). None of the epicotyls under 0.01mg/L of 2,4-D exhibited callogenesis.

Table 4.6 Callogenic response of different explants of two hybrid lines to different concentrations of 2,4-D in presence of 1mg/L of BA

Treatment with 2,4-D (mg/L)	Explant	explants that produced callus (%)	Callus		Explants with Necrosis (%)
			Type	Color	
0	Apical portion cotyledon	0.0	-	-	100.0
	Basal portion cotyledon	0.0	-	-	100.0
	Epicotyl	50.0	fr, nd,comp	br	50.0
	Radicle	83.3	comp	br, ye	83.3
	Hypocotyl	85.7	fr, nd	br, ye	85.7
0.01	Apical portion cotyledon	12.5	fr	ye, wh	25.0
	Basal portion cotyledon	12.5	-	-	62.5
	Epicotyl	0.0	-	-	62.5
	Radicle	50.0	fr, nd	wh, br	62.5
	Hypocotyl	85.7	nd	gr, br	71.4
0.05	Apical portion cotyledon	0.0	-	-	75.0
	Basal portion cotyledon	62.5	fr	br	87.5
	Epicotyl	75.0	fr, comp	light gr, wh	50.0
	Radicle	75.0	fr, nd	br, ye,	62.5
	Hypocotyl	87.5	fr, nd	wh, ye	75.0
0.1	Apical portion cotyledon	12.5	-	br,	50.0
	Basal portion cotyledon	25.0	fr	gr clar, ye	62.5
	Epicotyl	75.0	fr, nd	br,	75.0
	Radicle	100.0	nd	ye, br	62.5
	Hypocotyl	83.3	nd	br, ye, wh	83.3

fr = friable, nd = nodular , comp = compact, br =brownish, ye = yellowish, wh = whitish, gr = greenish

In absence of 2,4-D (0mg/L of 2,4-D and 1mg/L of BA), half of epicotyl explants (50%) and most of the explants from radicle (83.3%) and hypocotyl (85.7%) produced callus. On the other hand, cotyledon explants (apical and basal portions) were not induced to produce calli with this treatment. They did, however, produce callus in presence of 0.01mg/L of 2,4-D, as did the explants

from radicles (50%) and hypocotyls (85.7%). In contrast, epicotyl explants treated with 0.01mg/L of 2,4-D did not proliferate callus; instead, they became clorotic and hyperhydrated. A concentration of 0.05mg/L of 2,4-D did not induce callogenesis in explants from the apical portion of cotyledons, but it did produce some of the highest percentages in the other types of explants (basal portions of cotyledons, 62.5%; epicotyls, 75%; radicles, 75%; and hypocotyls, 87.5%). Finally a 2,4-D concentration of 0.1mg/L induced callogenesis in every type of explant, including a rate of 100% in the case of radicles.

In general, the calli produced with or without 2,4-D grew from the cut-zones, and the degree to which calli covered each explant varied according to the type of explant. In all treatments where epicotyls produced callus (0, 0.05 and 0.1mg/L of 2,4-D), calli formed only on basal zones (Figure 4.5a). On the other hand, in the case of radicle and hypocotyl explants, callus growth covered a third part of each explant when cultured in 0mg/L or 0.01mg/L of 2,4-D, and two thirds or more of each explant, when in 0.05 and 0.1mg/L of 2,4-D. Callus grew only from the cut-zones of apical and basal portions of cotyledons cultured with 0.01mg/L of 2,4-D and covered approximately one third or less of these explants in presence of 0.05 and 0.1mg/L of 2,4-D.

Necrosis always started at the cut-zones of the explants affected (60.7%) (Table 4.6). Some apical and basal cotyledon explants exhibited small necrotic zones in the surface of the explant as well, which finally invaded the cotyledon. The totality of cotyledons explants (apical and basal) suffered necrosis in

absence of 2,4-D, while the lowest percentage of necrosis in these explants was observed in presence of 0.01mg/L of 2,4-D. In epicotyl, radicle and hypocotyl explants, necrosis was restricted to the cut-zones, and most of the explants were able to proliferate calli. For epicotyl explants the highest percentage of necrosis was observed in presence of 0.1mg/L of 2,4-D (75%), while in 0, 0.01 and 0.05mg/L of 2,4-D necrosis was 50, 62.5%, and 50%, respectively. The highest amount of necrosis in radicle explants was observed in cultures without 2,4-D (83.3%), while in treatments containing 0.01, 0.05 and 0.1mg/L of 2,4-D, 62.5% of these explants developed necrosis. Finally, hypocotyl explants always showed high percentages of necrosis (ranging from 71.4% to 85.7%), but in contrast to cotyledonary explants, this did not hinder their ability to form calli.

The three types of callus (nodular, friable and compact) were observed in presence of the different concentrations of 2,4-D. Friable callus with yellowish, whitish and brownish coloration was detected in cotyledon explants in the presence of 0.01, 0.05 and 0.1mg/L of 2,4-D. Epicotyl explants developed compact and brownish callus without 2,4-D, while callus was friable and nodular, with brownish and whitish coloration in presence of 0.05 and 0.1mg/L of 2,4-D (epicotyls did not form calli under 0.01mg/L of 2,4-D). Around of 34% of the surviving epicotyl explants developed buds, both lateral and apical, at the cut-zone.

Radical explants developed compact calli in treatment without 2,4-D, while friable and/or nodular calli were produced in presence of any concentration of

2,4-D. Nodular calli covering half of the radical explants were formed with 0.1 mg/L of 2,4-D. Furthermore, roots were formed in all treatments of radicle explants. In presence of 0 and 0.01mg/L of 2,4-D, the roots originated from cut-zones (Figure 4.5b), whereas under 0.05 and 0.1mg/L of 2,4-D, roots derived from different zones of the calli, and they in turn formed callus at their tips or at their bases (Figure 4.5c).

Finally, hypocotyls developed nodular calli in presence of 0.01 and 0.1 mg/L of 2,4-D and callus with friable and nodular zones in 0 and 0.05mg/L of 2,4-D. Under 0 and 0.01mg/L of 2,4-D, this type of explant formed callus only at the cut-zones and on parts of the explant that made contact with the medium. In contrast, the hypocotyl explants in 0.05mg/L and 0.1mg/L of 2,4-D were entirely covered by callus. In this last treatment, globular structures were observed (Figure 4.5d).

In summary, it was observed that under 0.1mg/L of 2,4-D and 1mg/L of BA a higher proliferation of nodular callus was obtained, and most of the surface of the explants was free of necrosis. For this reason, this treatment was selected for the production and maintenance of nodular calli used as primary explants for further experiments.

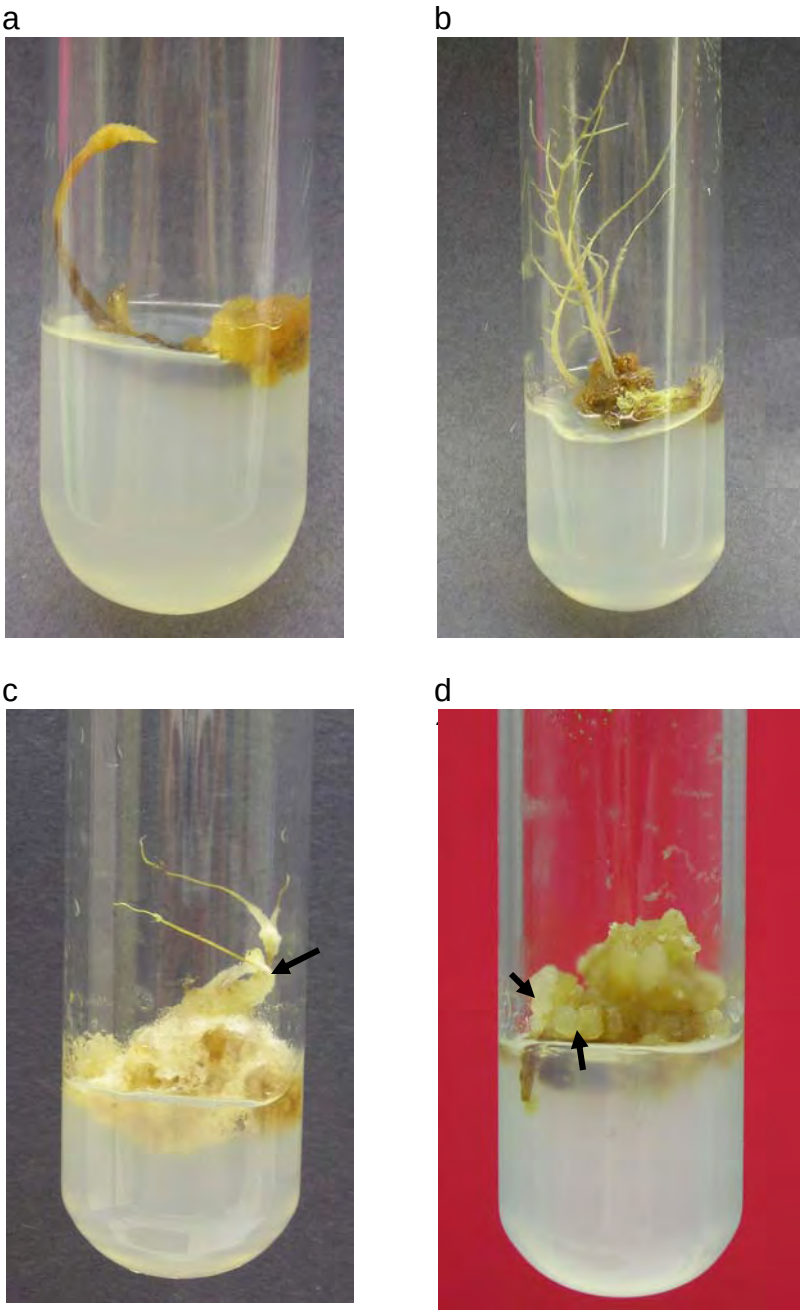

Figure 4.5. Response of explants in presence of different concentrations of 2,4-D. a) without 2,4-D, epicotyl explant with callus in the basal zone. b) In 0.01mg/L of 2,4-D, it was evident the production of both, callus and roots in the cut-zone of radical explants. c) Callus produced form radical explant in presence of 0.1mg/L of 2,4-D. Note callus formation on roots. d) Nodular callus produced on hypocotyl explants under 0.1mg/L of 2,4-D. Observe the presence of globular structures (arrows). Images a to c) correspond to hybrid line 155, while image d) corresponds to hybrid line 133

4.3.2 Callogenic Effect of 2,4-D at 29°C

The production of callus was apparent in the treatments with 2,4-D incubated at 29°C. The lowest percentage of explants developing callus was observed in treatments with 0 and 0.01mg/L of 2,4-D, and the highest percentage of explants with calli was obtained at 2,4-D concentrations of 0.1mg/L. Apical portions of cotyledons and radicle explants did not produce callus in 0 mg/L of 2,4-D, while basal portions of the cotyledons (12.5%), epicotyl (25%) and hypocotyl (28.6%) explants developed small calli (Table 4.7).

In the presence of 0.01mg/L of 2,4-D, only 12.5% of every type of explant developed calli (except for apical portion of cotyledons, where callus formation did not occur). In contrast, under concentrations 0.05 and 0.1mg/L of 2,4-D epicotyl, radicle and hypocotyl explants resulted in considerably higher percentages of callus formation, particularly epicotyls and hypocotyls under 0.1mg/L, in which callogenesis occurred in 100% of the explants.

Calli always formed at the cut-zones of the basal portions of cotyledons, epicotyl, radicle and hypocotyl explants cultured with 0 and 0.01mg/L of 2,4-D. Similarly, in presence of 0.05 and 0.1mg/L of 2,4-D, cotyledon explants (both, apical and basal) developed small calli only at the cut zone. However, epicotyls, radicles and hypocotyls cultured at higher hormone concentrations proliferated calli on most of the surface of the explant, especially those in 0.1mg/L of 2,4-D.

The calli obtained were friable and nodular, particularly those obtained in hypocotyl explants, with yellowish, whitish and brownish coloration.

Table 4.7 Callogenic response of different explants of two hybrid lines to different concentrations of 2,4-D at 29°C

Treatment with 2,4-D (mg/L)	Explant	% explants that produced callus	Callus		Explants with Necrosis (%)
			Type	Color	
0	Apical portion cotyledon	0	-	-	100.0
	Basal portion cotyledon	12.5	-	-	75.0
	Epicotyl	25	-	ye, br	25.0
	Radicle	0	-	-	12.5
	Hypocotyl	28.6	nd	br	0.0
0.01	Apical portion cotyledon	0	-	-	100.0
	Basal portion cotyledon	12.5	fr	ye, wh	100.0
	Epicotyl	12.5	-	-	37.5
	Radicle	12.5	-	-	87.5
	Hypocotyl	12.5	-	-	12.5
0.05	Apical portion cotyledon	25.0	-	wh,	75.0
	Basal portion cotyledon	25.0	nd	wh	62.5
	Epicotyl	87.5	fr	br,	50.0
	Radicle	87.5	fr, nd	wh, ye, br	25.0
	Hypocotyl	83.3	fr, nd	wh, ye	50.0
0.1	Apical portion cotyledon	25.0	-	br	37.5
	Basal portion cotyledon	37.5	fr	br, wh	75.0
	Epicotyl	100	fr	wh, ye, br	50.0
	Radicle	75.0	fr	wh, ye	37.5
	Hypocotyl	100	fr, nd	br, wh, ye	57.1

fr = friable, nd = nodular , comp = compact, br = brownish, ye = yellowish, wh = whitish, gr = greenish

As in other experiments, different explants behaved differently under different treatments. All epicotyl explants grew vertically (from the original horizontal

position) and became etiolated (Figure 4.6a). In 0.01mg/L of 2,4-D they also appeared hyperhydrated, and with 0.05 and 0.1mg/L of 2,4-D, they produced roots and few calli along the explant (Figure 4.6b). Radicle explants developed roots in all treatments, while hypocotyls developed roots only when cultured in 0.1mg/L of 2,4-D. Roots derived from radicle and hypocotyl explants were long and branched with 0 and 0.01mg/L of 2,4-D, whereas those produced in 0.05 and 0.1mg/L of 2,4-D were shorter, unbranched, and presented calli at their basal zone or at the root-tips (Figure 4.6c). In hypocotyl explants roots were observed emerging from globular structures (Figure 4.6d).

Necrosis occurred in all the types of explants cultured and for all the treatments, excepting hypocotyls cultured without 2,4-D. In the case of apical and basal cotyledonary portions, necrosis was evident at the cut-zones and also in small superficial zones of the cotyledons, and it progressively invaded the entire explant. Apical portions of cotyledon explants showed the highest percentages of necrosis (75% and 100%) in presence of 0, 0.01 and 0.05mg/L of 2,4-D, while basal portions of cotyledons exhibited similar percentages when cultured in 0, 0.01 and 0.1mg/L of 2,4-D. Only 37% of apical portions of cotyledons had necrotic zones in presence of 0.1mg/L of 2,4-D.

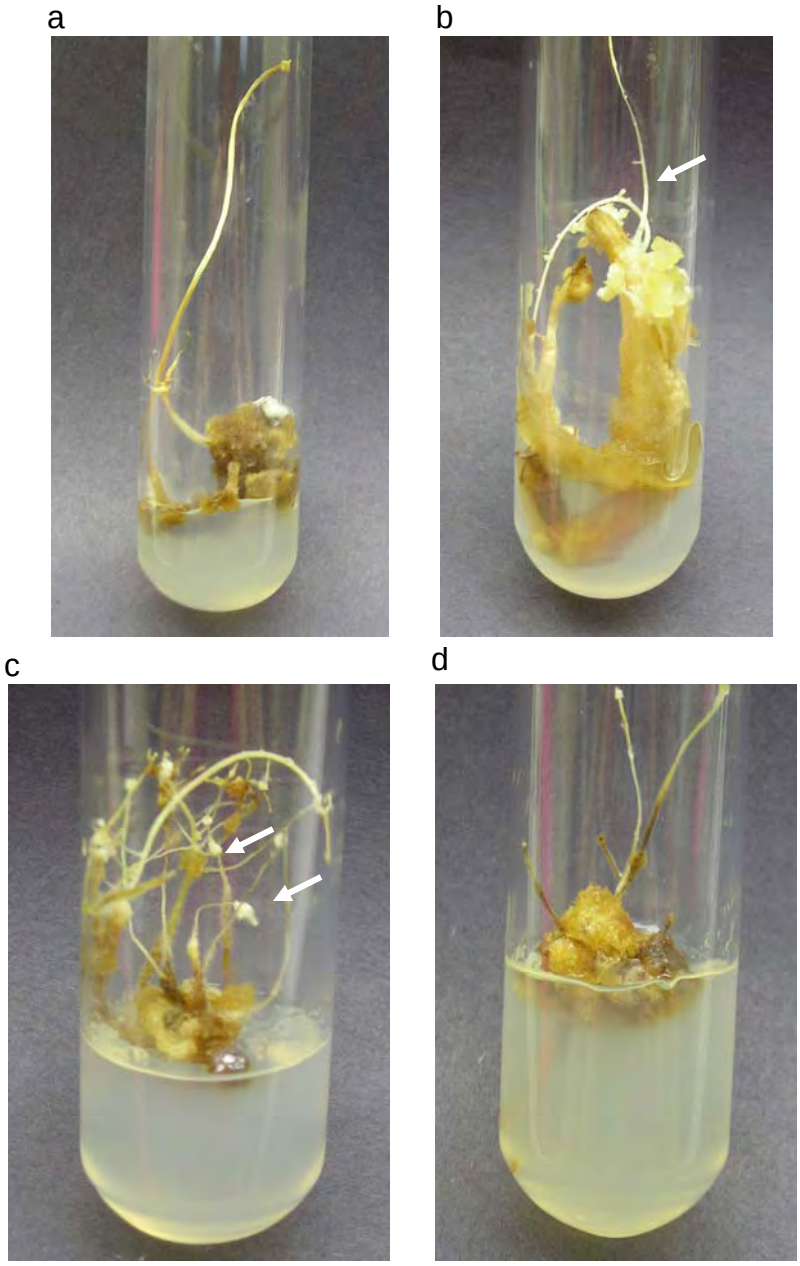

Figure 4.6 Response of explants to different concentrations of 2,4-D at 29°C. a) Elongation of epicotyls in presence of 2,4-D. Observe the length and etiolation of explants. b) Production of roots and callus in epicotyls with 0.1mg/L of 2,4-D. c) Radicle explant cultured in presence of 0.05mg/L of 2,4-D. Note the proliferation of roots and calli in root-tips. d) Roots in globular structures observed in hypocotyl explants with 0.1mg/L of 2,4-D. Images a) and d) correspond to hybrid line 133 and images c) and d) to hybrid line 155

Epicotyl, radicle and hypocotyl explants also suffered necrosis, the cut-zones always being the first becoming affected. Necrosis observed in epicotyl and radicle explants eventually covered approximately one third of the explants' surface, while in hypocotyls necrosis remained restricted to the cut-zones. The percentage of epicotyls that became necrotic was lower for 0 and 0.01mg/L of 2,4-D (25 and 37.5%, respectively) than for 0.05 and 0.1mg/L of 2,4-D (50%). In radicles the lowest percentage of necrosis was observed without 2,4-D (12.5%) and the highest at 0.01mg/L of 2,4-D (87.5%). Finally, necrosis was not apparent in hypocotyl explants cultured without 2,4-D, whereas the percentage of explants with necrosis increased to 50% or more in the presence of 0.05mg/L and 0.1mg/L of 2,4-D.

4.4 Maturation of Nodular Callus

4.4.1 Effect of Air-drying

Nodular calli were air-dried for fifteen days and subsequently transferred to a medium supplemented with 0.1mg/L of 2,4-D and 1mg/L of BA. After 30 days of culture, it was evident that callus survival was not affected by air-drying. Interestingly, the percentage of explants with necrosis was higher in control (60.9%) than in the air-dried calli (33.3%) (Table 4.8). In all the cultures, necrosis affected less than one third of each individual callus.

Table 4.8 Response of calli air-dried for fifteen days and cultured in 0.1mg/L of 2,4-D and 1mg/L of BA

Treatment	Callus		Explants with Necrosis (%)
	Callus color	Texture	
Control	gr br, ye	nd, comp, fr	60.9
Air-drying	light br, wh	nd	33.3

fr = friable, nd = nodular, comp = compact, br = brownish, ye = yellowish, wh = whitish, gr = greenish

All the air-dried cultures proliferated light brownish and whitish nodular calli, whereas brownish, yellowish and greenish nodular, compact and friable calli were abundant in the control cultures. Globular structures developed in all air-dried calli, while in the control cultures such structures were only observed in nodular and nodular-friable calli (approximately 34% of explants) (Figure 4.7a). In both treatments (air-dried calli and non-air-dried calli) a root emerged from each globular structure. In two of the air-dried calli (5%) a flat, smooth, M-shaped structure developed, which grew vertically to an approximate size of 5mm. Electron microscopy images of these structures revealed embryo-like structures in torpedo or cotyledonary stages, embedded in the callus (Figure 4.7b), with both cotyledons and radicle zones. Other than this, there was no additional evidence of embryogenesis; all the cultures proliferated nodular callus with globular structures.

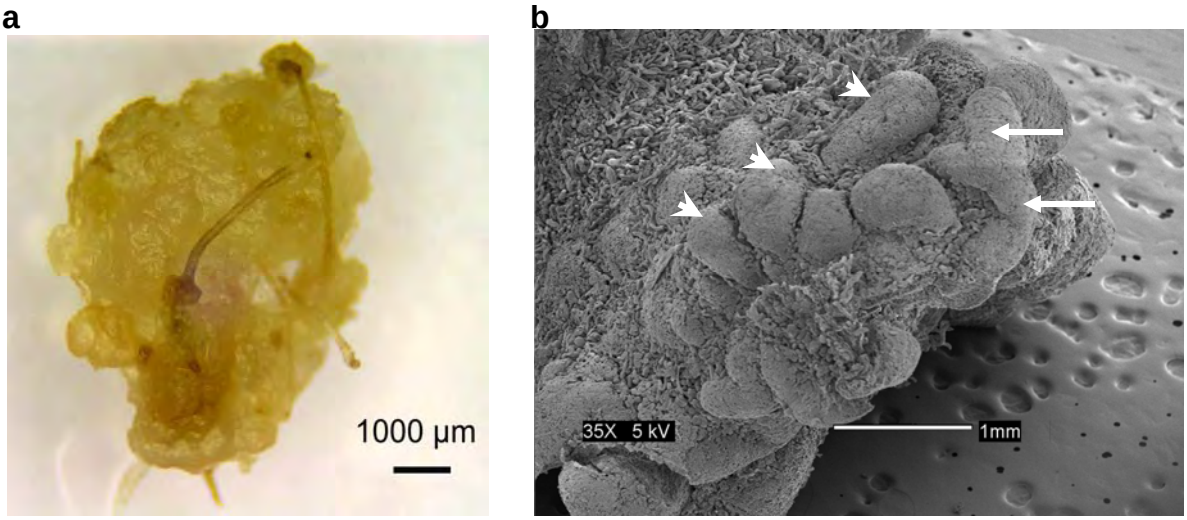

Figure 4.7 Air-dried cultures. a) Globular structures with elongated projection reminiscent of radicle. These structures were also in non-air-dried calli. b) Scanning electron micrograph showing detail of embryo-like structures (arrowheads) in air-dried calli. Arrows point to structures reminiscent of cotyledonary leaves and radicles of an embryo in the torpedo stage.

4.4.2 The Effect of Liquid Medium

Nodular calli from solid medium supplemented with 0.1mg/L of 2,4-D and 1mg/L of BA, were cultured in liquid medium with similar plant regulators' concentrations. After 30 days of culture in liquid medium, yellowish and whitish disaggregated calli were observed. A solid medium with 0.1mg/L of 2,4-D and 1mg/L of BA, used as a control for this experiment, produced nodular, friable and nodular-friable calli with brownish, yellowish and greenish coloration (Table 4.9). 65.2% of explants on this control treatment had necrotic areas in less than one third of the callus surface. In contrast, there was no necrosis in calli cultured in liquid medium.

Table 4.9 Response of callus to the effect of liquid medium

Type of medium	Callus		Explants with Necrosis (%)
	Color	type	
Solid (Control)	br, wh, gr, ye	Nd, fr	65.2
Liquid	Light br, wh	Disaggregated callus	0

fr = friable, nd = nodular , br =brownish, ye = yellowish, wh = whitish, gr = greenish

Approximately 60% of calli incubated in liquid medium produced brownish and spherical structures (Figure 4.8a). Examination of the calli using light microscopy, revealed globular and heart-shaped structures (Figure 4.8 b and c). An isolated case of an embryo with cotyledonary leaves and radicle apex, which reached 1-2 mm in length, was observed after 20 days of culture. Unpredictably, this embryo reversed to callus proliferation after 30 days of culture. Controls also proliferated calli with globular structures and roots, as shown in Figure 4.8a, in 26% of the cultures.

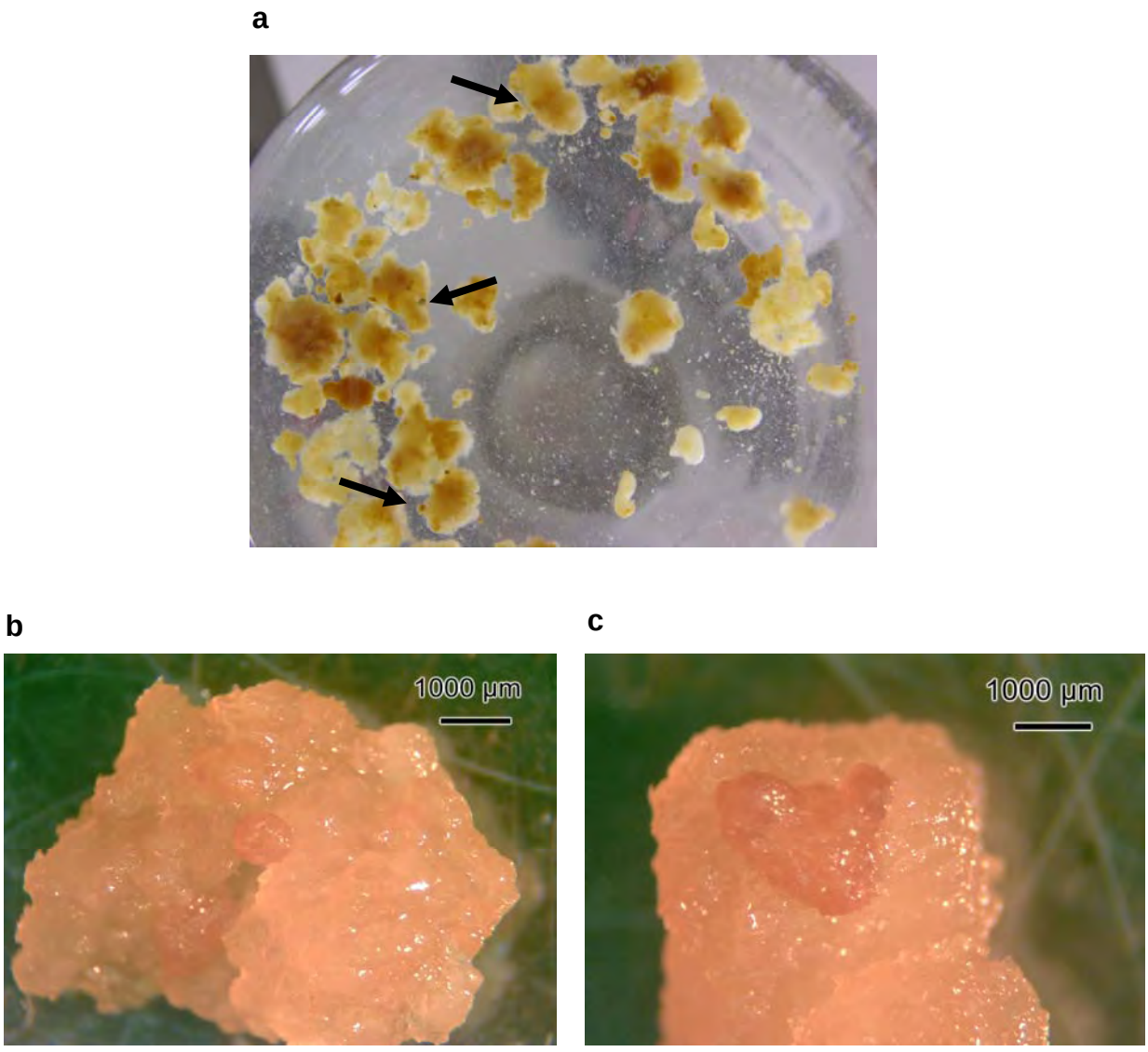

Figure 4.8 Liquid cultures in presence of 0.1mg/L of 2,4-D and 1mg/L of BA.
a) Disaggregated callus, note the spherical structures within callus. b) Globular stage.
c) Heart stage

4.4.3 The Effect of $AgNO_3$

Nodular calli in presence of $AgNO_3$ were analyzed after 30 days of culture, revealing characteristics that were similar to those of calli cultured without $AgNO_3$ (Table 4.10). However, there was a difference in terms of necrosis: in presence of $AgNO_3$ the percentage of explants with necrosis reached 27.3% covering, in every case, less than a third of each callus; while in the controls necrosis was not evidenced.

Table 4.10 Response of calli treated with $AgNO_3$

$AgNO_3$ (mg/L)	Callus		Explants with Necrosis (%)
	Color	Type	
0 (Control)	ye, br	nd	0
1.7	ye, br	nd,fr	27.3

fr = friable, nd = nodular , br =brownish, ye = yellowish, wh = whitish, gr = greenish

Globular structures, some of them with roots, appeared on both, experimental calli and controls. Additionally, cultures treated with $AgNO_3$ showed similar structures to those shown in Figure 4.7 (b and c), and others with acute apices (Figure 4.9).

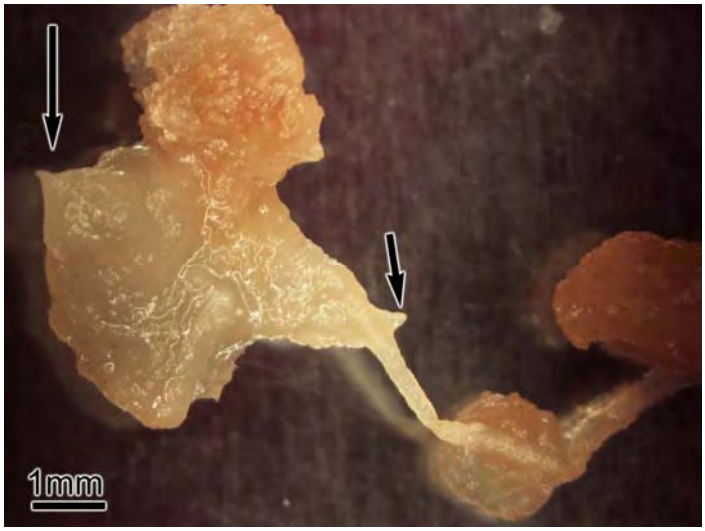

Figure 4.9 Structures observed in presence of $AgNO_3$. Note the acute apices of the structure

4.4.4 The Effect of Desiccation in Combination with $AgNO_3$

Nodular calli air-dried for 15 days and then transferred to media with or without $AgNO_3$, appeared similar in both treatments. The percentage of explants with necrosis was higher (60.9%) in calli cultured without $AgNO_3$ than in those with $AgNO_3$ (41.1%), as shown in Table 4.11. In all cases necrosis invaded less than one third of each individual callus.

Table 4. 11 Response of calli air-dried for 15 days and cultured with or without $AgNO_3$

Explant	$AgNO_3$ (mg/L)	Callus		Explants with Necrosis (%)
		Color	Type	
Air-dried calli	0 (Control)	br, ye	nd, fr	60.9
	1.7	Br, wh, ye, gr	nd	41.1

fr = friable, nd = nodular , br =brownish, ye = yellowish, wh = whitish, gr = greenish

In presence of the $AgNO_3$, nodular calli with brownish and yellowish coloration proliferated. On the other hand, without $AgNO_3$, both nodular and friable calli developed, which varied in color from whitish to yellowish, brownish and greenish (Table 4.11). In both treatments, calli presented globular structures. The treatment without $AgNO_3$ produced roots, which originated from the globular structures, as illustrated in Figure 4.10a. Spherical and embryo-like structures with cotyledonary leaves and radical apex, similar to those observed in section 4.4.1 were developed in presence of $AgNO_3$ ((Figure 4.10b).

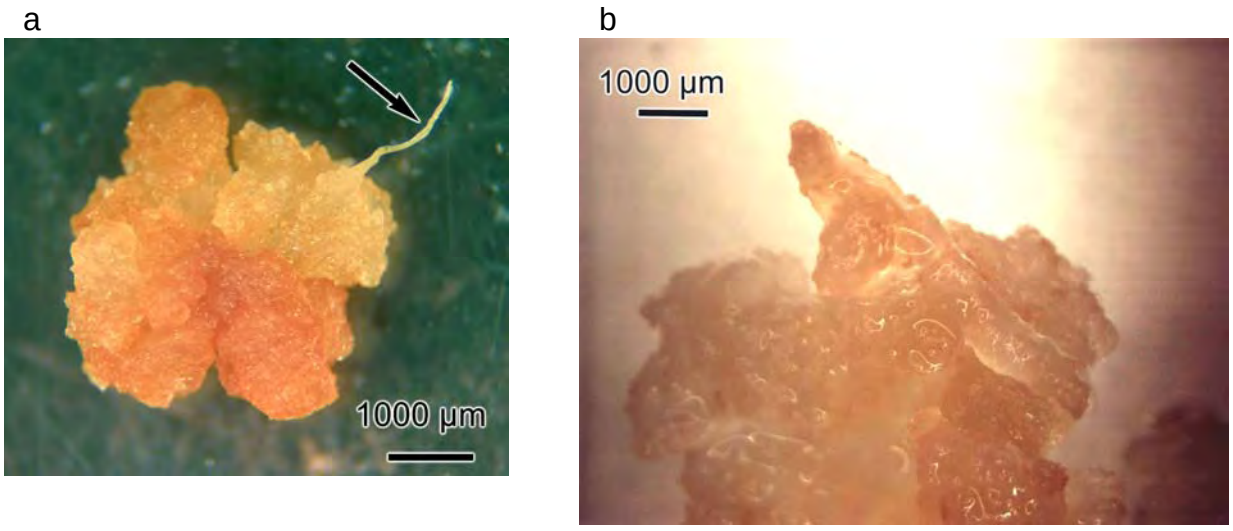

Figure 4.10 Structures observed assaying the effect of the dessication in combination with $AgNO_3$. a) Globular structures with roots without $AgNO_3$. b) embryo-like structure observed in presence of $AgNO_3$

4.4.5 The Effect of Liquid Medium and $AgNO_3$

Calli cultured in liquid versus solid media, both supplemented with 0.1mg/L of 2,4-D, 1mg/L of BA and 1.7mg/L of $AgNO_3$, showed morphological differences between them. While no necrosis occurred in calli growing in liquid media, 32% of the calli cultured in solid medium exhibited small areas of necrosis (Table 4.12). In addition, cultures on solid medium developed nodular callus with a brownish and yellowish color, while the liquid medium resulted in disaggregated callus with whitish and brownish color. Only the calli cultured in liquid media produced brownish structures, similar to those shown in Figure 4.8

Table 4. 12 Response of calli cultured in both liquid and solid media with $AgNO_3$

Consistence of medium	$AgNO_3$ (mg/L)	Callus		Explants with Necrosis (%)
		Color	Type	
Solid (control)	1.7	br, ye	nd	32.0
Liquid	1.7	wh,br	Disaggregated callus	0

fr = friable, nd = nodular , br =brownish, ye = yellowish, wh =whitish, gr = greenish

4.5 Organogenesis

4.5.1 Proliferation

After 30 days of culture in presence of 0.5mg/L of BA, lateral and adventitious buds (from the hybrid lines) did not proliferate and did not develop satisfactorily. Approximately, 50% of buds suffered necrosis. The older leaves from surviving buds became chlorotic and subsequently necrotic. Compact and chlorotic shoots developed after 60 days of culture (Figure 4.11a).

When buds were transferred to basal medium with 2.25mg/L of BA and 1.7mg/L of $AgNO_3$, both adventitious and lateral buds proliferated, and after 30 days of incubation, from 3 - 10 new buds had developed from each of the initial buds (Figure 4.11b). Necrosis often affected almost half of each explant, but did not limit the production of buds. When buds were subcultured in a medium with similar composition, there was a reduction in the extent of necrosis. In this instance, there was a high proliferation of buds (up to ten new buds from each subcultured bud), and development of leaves was also observed (Figure 4.11c).

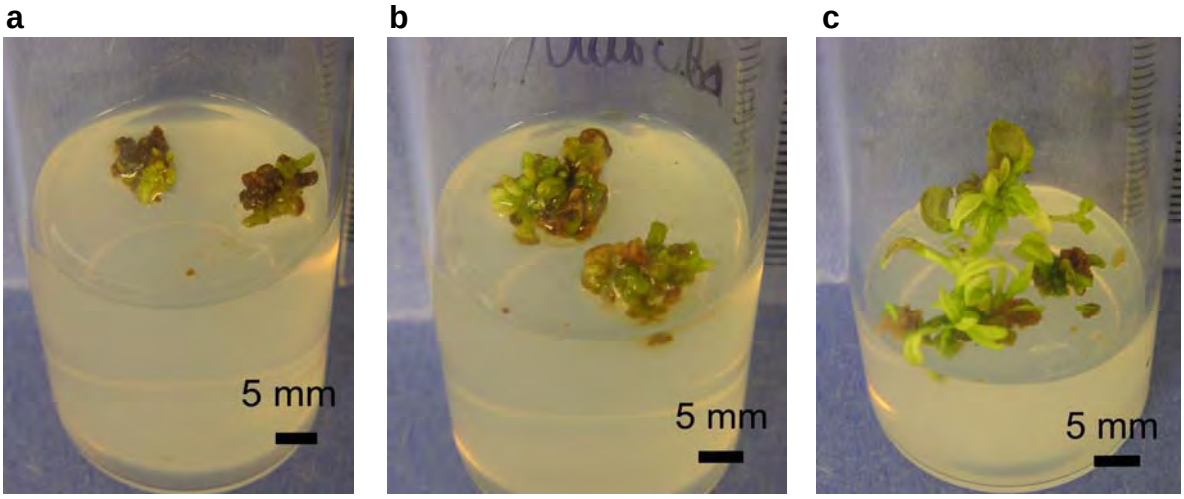

Figure 4.11. Proliferation of buds. a) buds cultured in 0.5mg/L of BA. In this treatment necrotic tissues surrounding the buds were observed. b) and c). The presence of BA and $AgNO_3$ favored the proliferation of buds. After subculturing in the same medium development of leaves were observed (c)

4.5.2 Elongation

Clumps of buds were transferred to the elongation medium of 2.25mg/L of BA, 1.7mg/L of $AgNO_3$ and and 1.5 mg/L GA_3 proposed by Carvalho et al (2000), in which the buds proliferated. After 30 days of culture under these conditions, shoots developed and elongated in 29% of the cultures. Shoots reached up to 6cms, with two or three nodes. The remaining 71% of the cultures did not develop shoots. Nonetheless, in these the production of multiple buds was evident near the base of developing shoots (Figure 4.12). In general, the appearance of shoots and buds in this treatment was normal. Necrosis was observed in all of the cultures, but it did not limit the production of buds or the development of shoots.

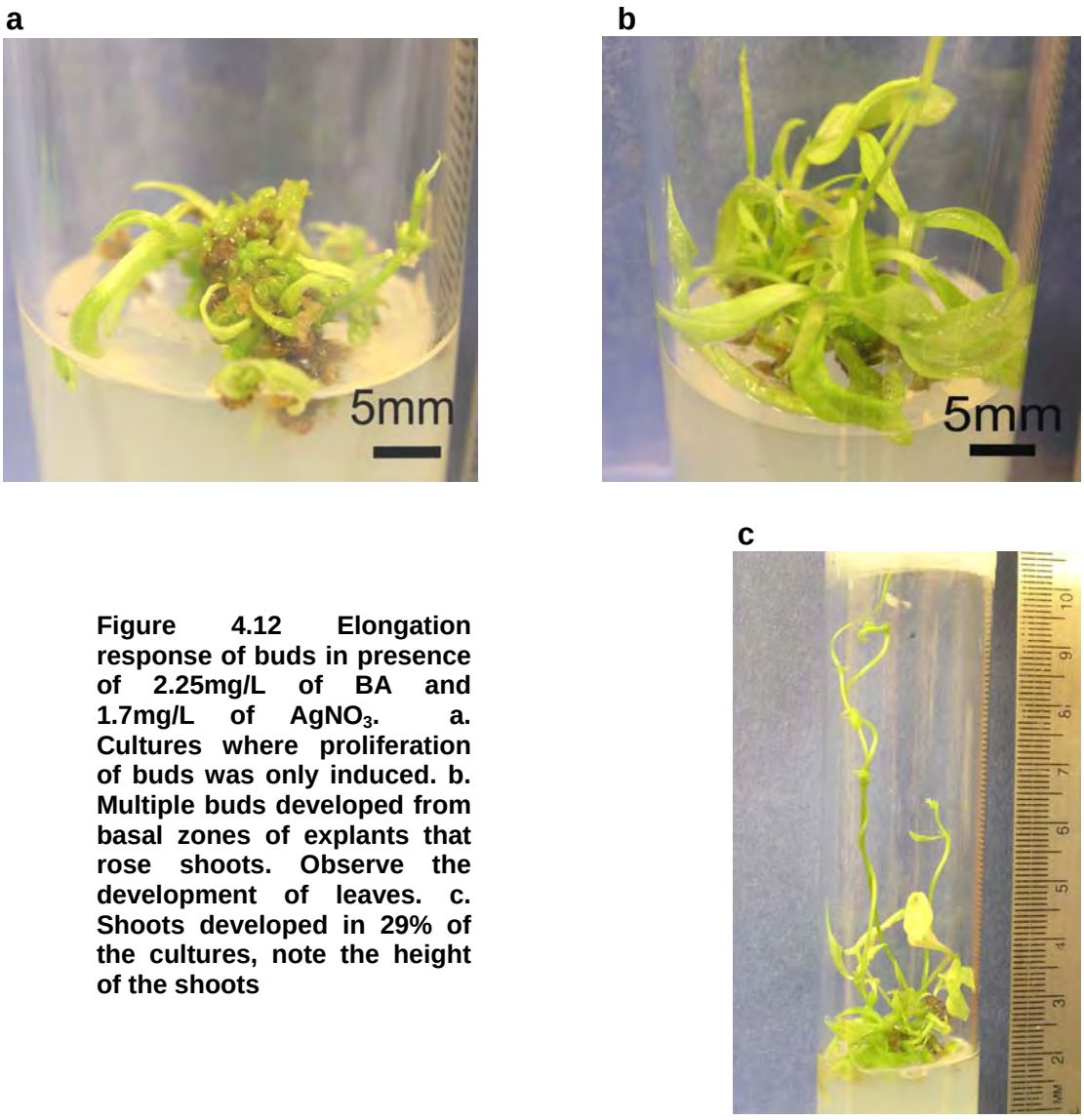

Figure 4.12 Elongation response of buds in presence of 2.25mg/L of BA and 1.7mg/L of $AgNO_3$. a. Cultures where proliferation of buds was only induced. b. Multiple buds developed from basal zones of explants that rose shoots. Observe the development of leaves. c. Shoots developed in 29% of the cultures, note the height of the shoots

4.5.2 Rooting

After 30 days of culture in basal medium supplemented with different combinations of BA, GA_3, NAA, $AgNO_3$, it was observed a limited production of

shoots rooted as illustrated in Figure 4.13. Only one shoot cultured with 0.2mg/L of NAA and 1.7mg/L of $AgNO_3$ developed three roots. The roots produced were brown and thick, with 3mm of length (see Figure 4.13 b). On the other hand, the treatments in presence of BA, GA_3 and NAA combinations did not induced rooting of shoots.

The elongation of shoots was also repressed in all the treatments, contrary to the proliferation of buds, which were produced in the basal zone of the shoots. The highest proliferation of buds (more than 10 per explant) was observed in all the treatments with $AgNO_3$, mainly in treatments where the NAA was increased (2.25mg/L of BA, 1.5mg/L of GA_3, and 0.4mg/L of NAA) and with high levels of BA (4.5mg/L of BA, 1.5mg/L of GA_3 and 0.2mg/L of NAA). The buds produced in this last treatment presented leaves with necrotic apices. The lowest production of buds (3 to 4 per explant) was obtained in treatment without $AgNO_3$ and in presence of 2.25mg/L of BA, 1.5mg/L of GA_3 and 0.2mg/L of NAA.

All shoot cultures presented necrosis. In treatments with $AgNO_3$, necrosis covered less than one third of the explants, while in absence of $AgNO_3$ the entire explants were necrotic (Figure 4.13f).

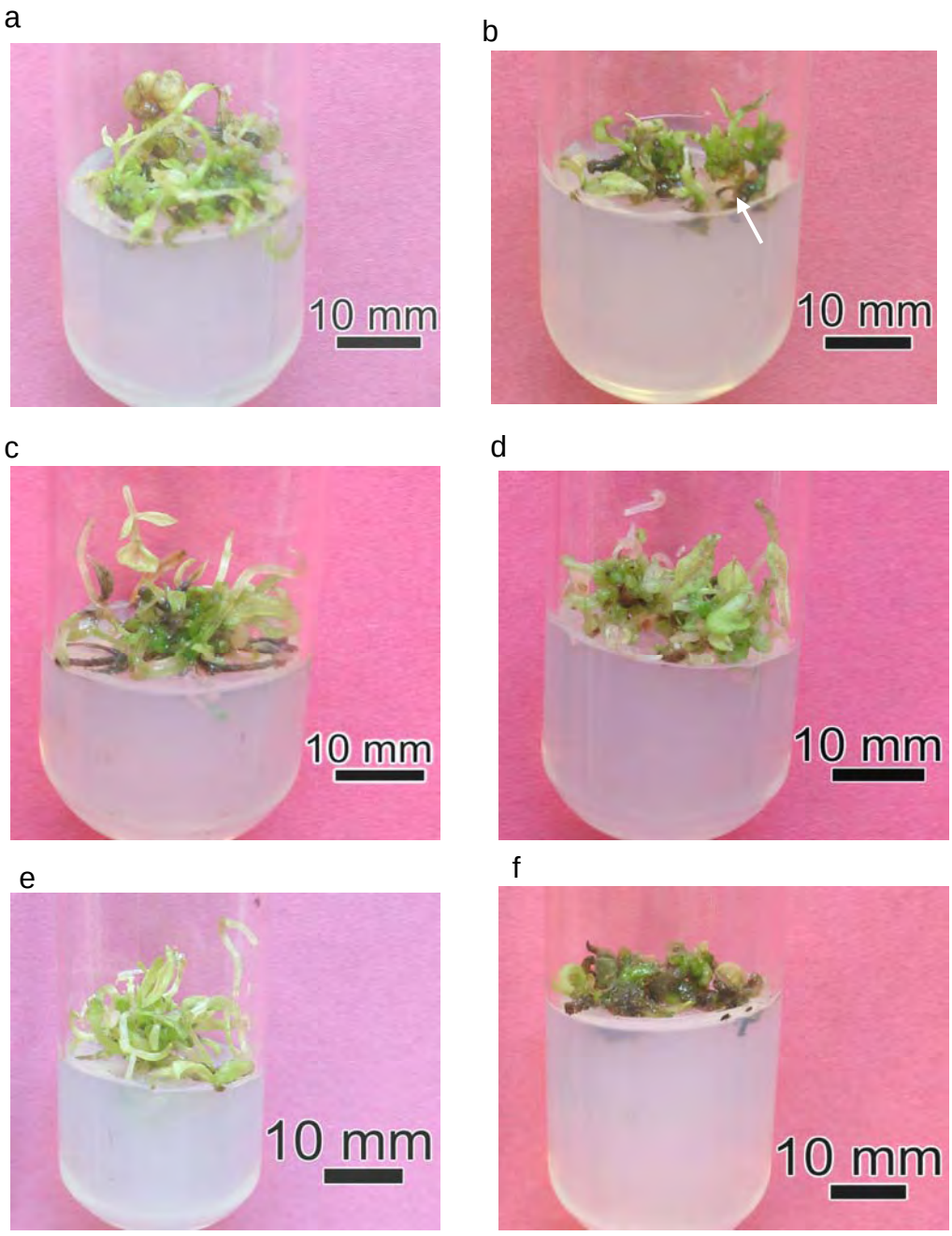

Figure 4.13 Response of shoots cultured in rooting treatments: a) In presence of 2.25mg/L of BA, 1.5mg/L of GA_3 and 1.7mg/L of $AgNO_3$ (control treatment). b) 0.01mg/L of NAA and 1.7mg/L of $AgNO_3$. Arrow showed a root developed. c) In presence of 2.25mg/L of BA, 1.5mg/L of GA3, 0.2mg/L of NAA and 1.7mg/L of $AgNO_3$. d) with 2.25mg/L of BA, 1.5mg/L of GA3, 0.4mg/L of NAA and 1.7mg/L of $AgNO_3$. e) with 4.5mg/L of BA, 1.5mg/L of GA_3, 0.2mg/L of NAA and 1.7mg/L of $AgNO_3$. f) with 2.25mg/L of BA, 1.5mg/L of GA_3 and 0.2mg/L of NAA (without $AgNO_3$). Observe the proliferation of buds and the necrosis level of the explants.

4.6 Scanning Electron Microscopy

All chemically fixed and cryo-fixed calli presented cell distortion to a considerable degree. Nevertheless, these treatments allowed some observations and inferences to be made about the structural organization of calli.

Chemical fixation, as well as the use of liquid propane or liquid nitrogen as cryogens for rapid freezing, allowed the preservation of *some* turgent cells, which appeared globose in shape (Figures 4.14 and 4.15). This type of morphology was frequently seen in chemically fixed calli (Figure 4.14 a and b), while in rapidly frozen samples, it was more easily seen in the non-DMSO-pretreated calli (Figures 4.14c and d, and 4.15 a and b), than in those pretreated with DMSO, where cell collapse was ubiquitous (Figures 4.14e and f, and 4.15c and d). In calli not pretreated with DMSO and fixed by rapid freezing, a superficial secretion or flattened layer could also be discerned, which appeared to cover most of the surface of the calli. Areas of the callus not covered by this material appeared to be formed by filiform cells (Figures 4.14d and 4.15b).

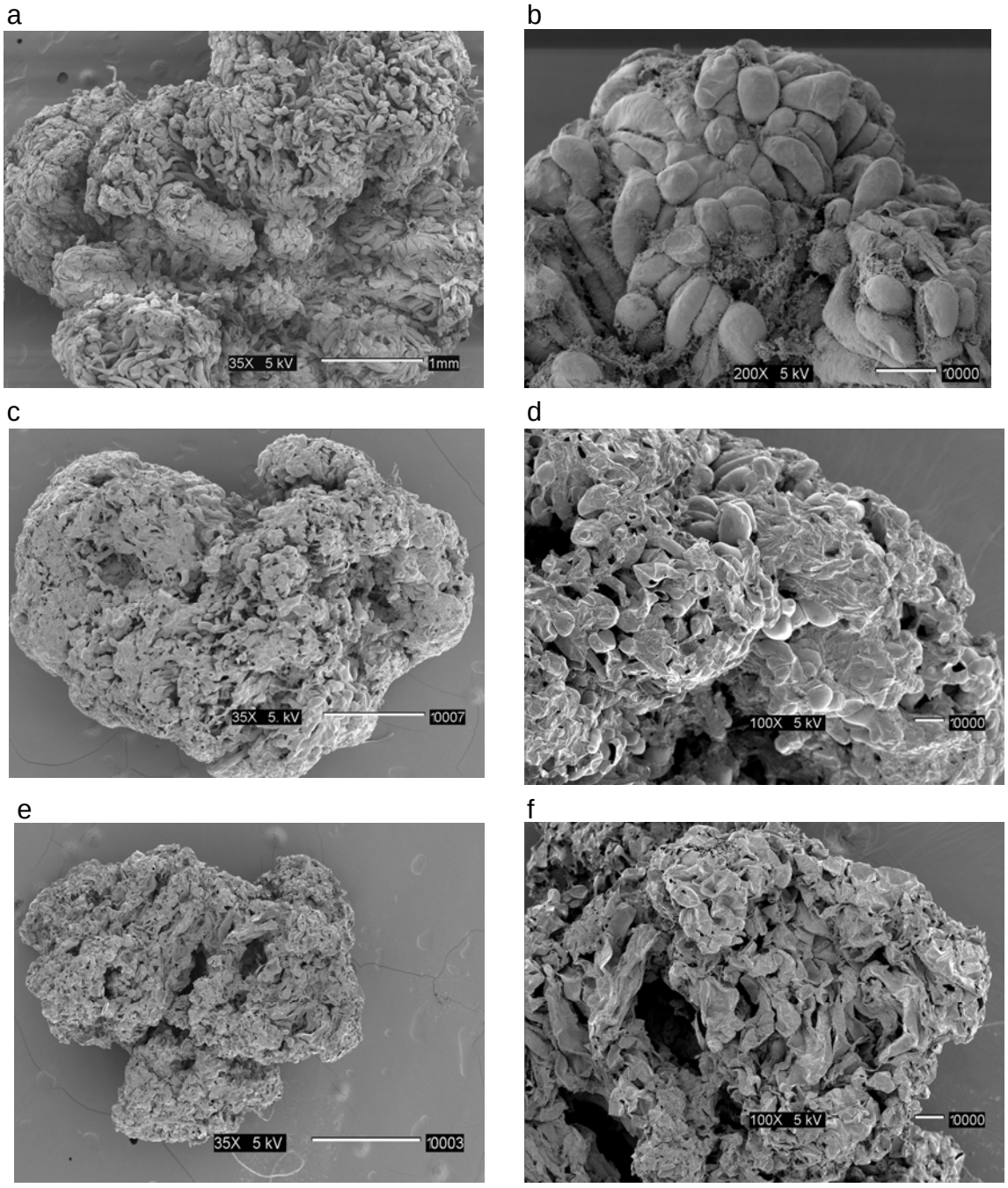

Figure 4.14 Chemically fixed (a and b) and rapidly cryo-fixed (c to f) calli. c) to f) Calli fixed in liquid propane. c) and d) non-DMSO-pretreated. Note the secretion or flattened superficial layer. e) and f) callus treated with DMSO.

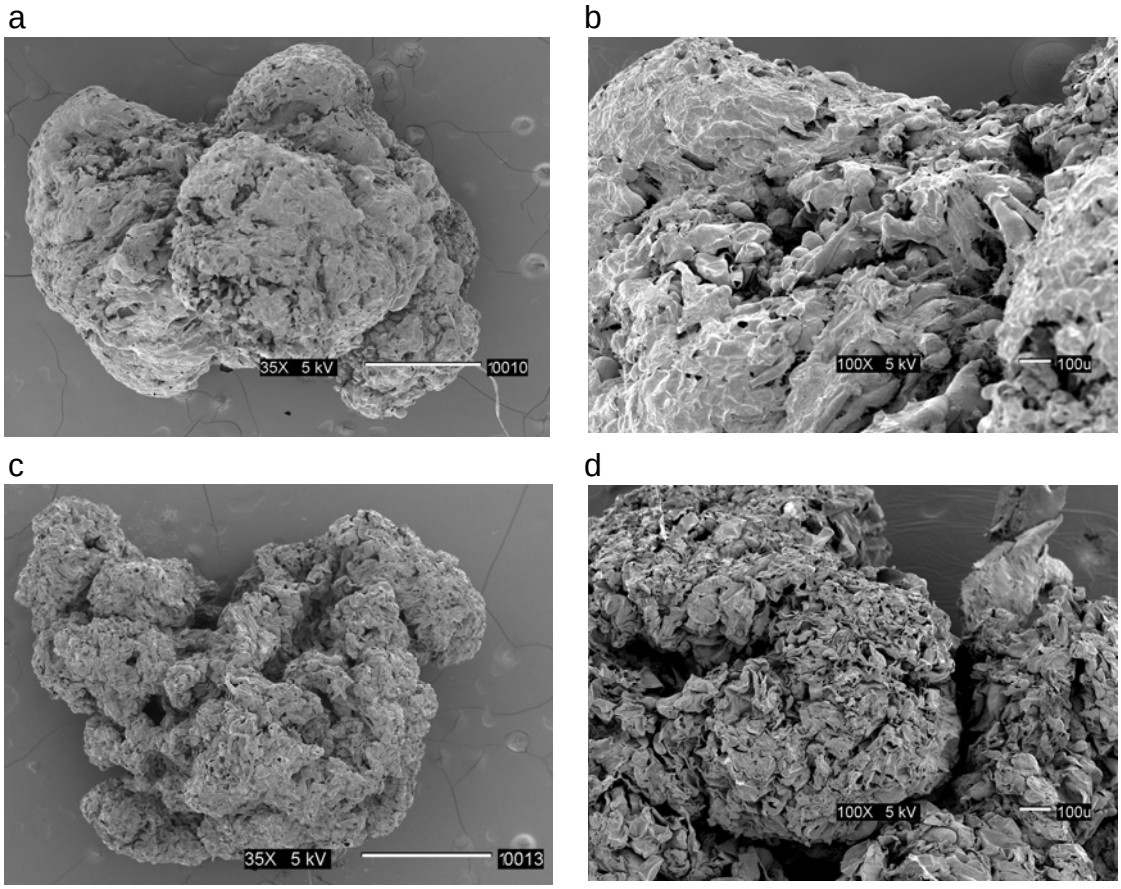

Figure 4.15 Calli rapidly cryo-fixed in liquid nitrogen. (a and b) non-DMSO-pretreated. Note the secretion or flattened superficial layer.(c and d) callus treated with DMSO.

Calli fixed by slow freezing showed *many* turgent cells, especially those that were not pretreated with DMSO (Figure 4.16 a and b). As in rapidly cryofixed calli, a flattened superficial layer or secretion was observed, which was apparently more homogeneous in calli not pretreated with DMSO (Figure 4.16a) than in those with DMSO (Figure 4.16c).

Finally, calli fixed by slow cooling in an ethanol bath followed by immersion in liquid nitrogen, became totally collapsed, whether pretreated with DMSO or not

(Figure 4.17). As in previous experiments, a flattened superficial layer was noticed in some areas of non-DMSO-pretreated calli (Figure 4.17a and b).

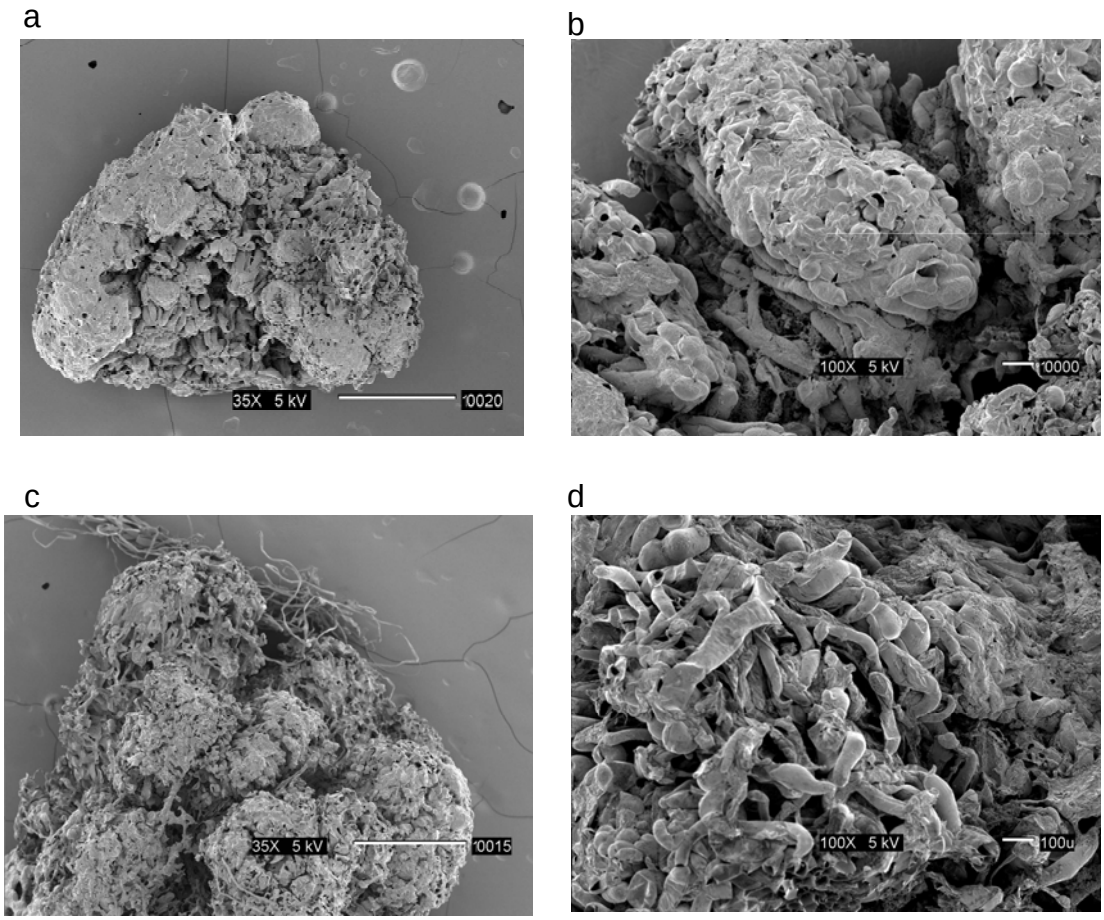

Figure 4.16. Calli fixed by slow freezing. (a and b) non-DMSO-pretreated callus. Observe the turgent cells. (c and d) DMSO-pretreated callus.

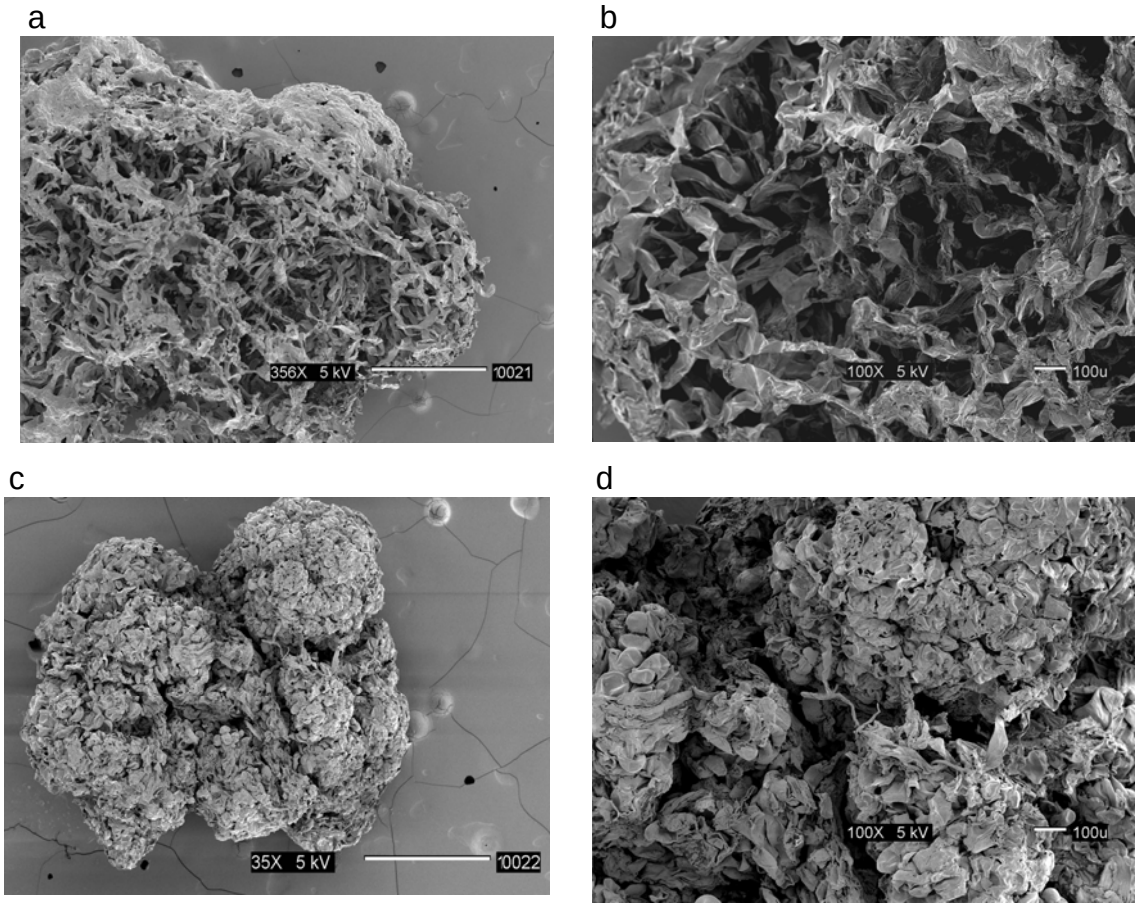

Figure 4.17 Calli fixed by slow cooling. (a and b) non-DMSO-pretreated callus. Note the secretion or flattened superficial layer. (c and d) DMSO-pretreated callus.

When ten additional samples were fixed either by rapid freezing (immersion in liquid nitrogen) or by slow freezing in a freeze drying apparatus, results were inconsistent with respect to our preliminary observations. Most cells appeared collapsed in all samples, as shown in Figure 4.18. We did see, however, *some* turgent cells in nine out of ten calli fixed by rapid freezing, and three of the seven calli processed by slow freezing (Figure 4.18 a to d). On the other hand, the flattened surface layer or secretion seen in previously studied samples was

confirmed. It was seen in all rapidly frozen calli (Figure 4.18 a), and in the majority (70%) of the slowly frozen calli (figure 4.18c).

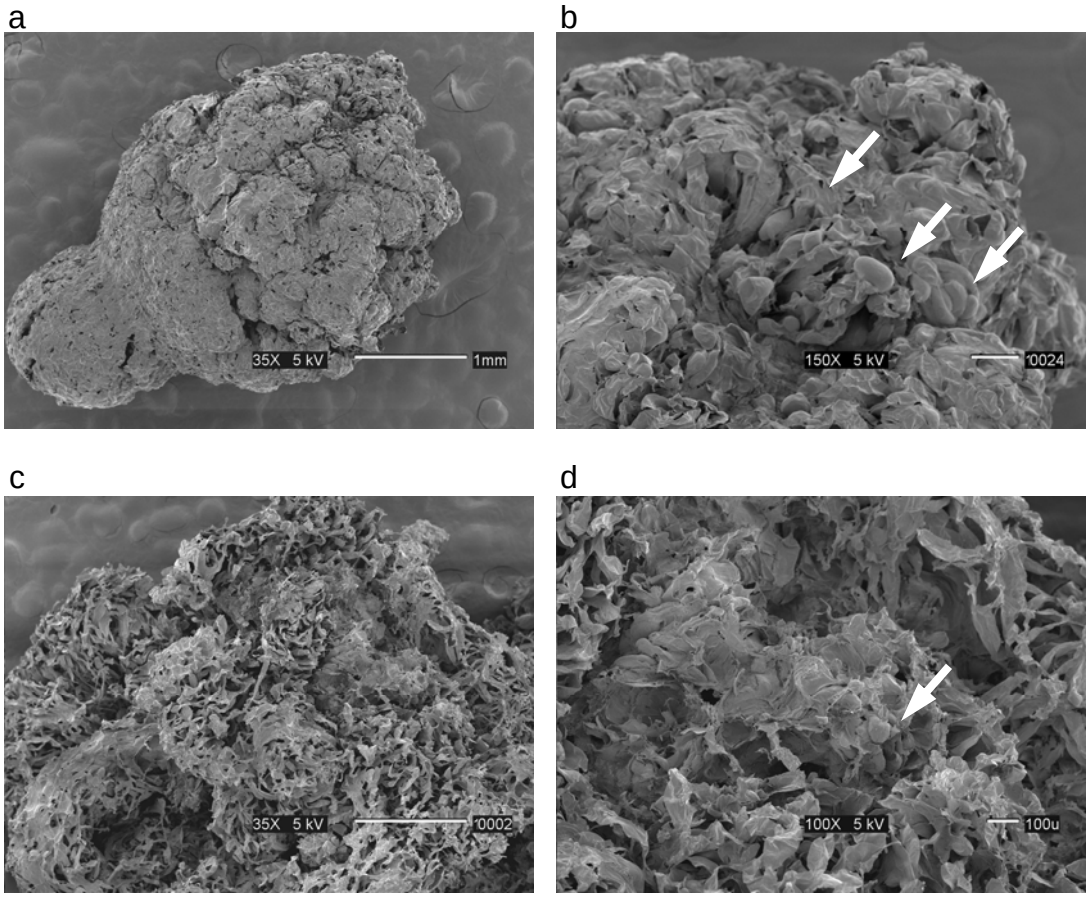

Figure 4.18 Additional calli fixed by rapid freezing with liquid nitrogen and slow freezing. a)Callus fixed by rapid freezing with liquid nitrogen. b) Detail of callus shown in a). c) callus fixed by slow freezing. d) Detail of callus shown in c). Note the few turgent cells among the collapsed cells (arrows).

In spite of the substantial degree of collapse of cells in these experiments, some inferences can be made about the structural composition of the calli. It appears that some calli are entirely formed by filamentous cells, arranged relatively loosely with air spaces between them (Figures 4.14f, 4.15c, 4.16c,

4.17b, 4.18d); while others are characterized by globose cells in a compact arrangement (Figure 4.14b, and d 4.16a, 4.18b). A third type of callus comprises both, zones with loosely arranged filamentous cells, and areas containing a more compact arrangement of globose cells (Figure 4.15a and c, 4.16b and d, 4.17d).

The fresh samples observed under low vacuum SEM became totally collapsed within less than 5 minutes of observation. However, we were able to photograph a small compact array of globose cells before they collapsed (Figure 4.19a and b). A few of these cells remained turgent for up to about 10 minutes. As in cryofixed calli, different types of cell morphology and arrangement were observed, including filiform and globose cells, either loosely (Figure 4.19c and d) or compactly arranged (Figure 4.19e). In addition, we observed a structure emerging from one of the calli, which had an arrangement reminiscent of a root (Figure 4.19f).

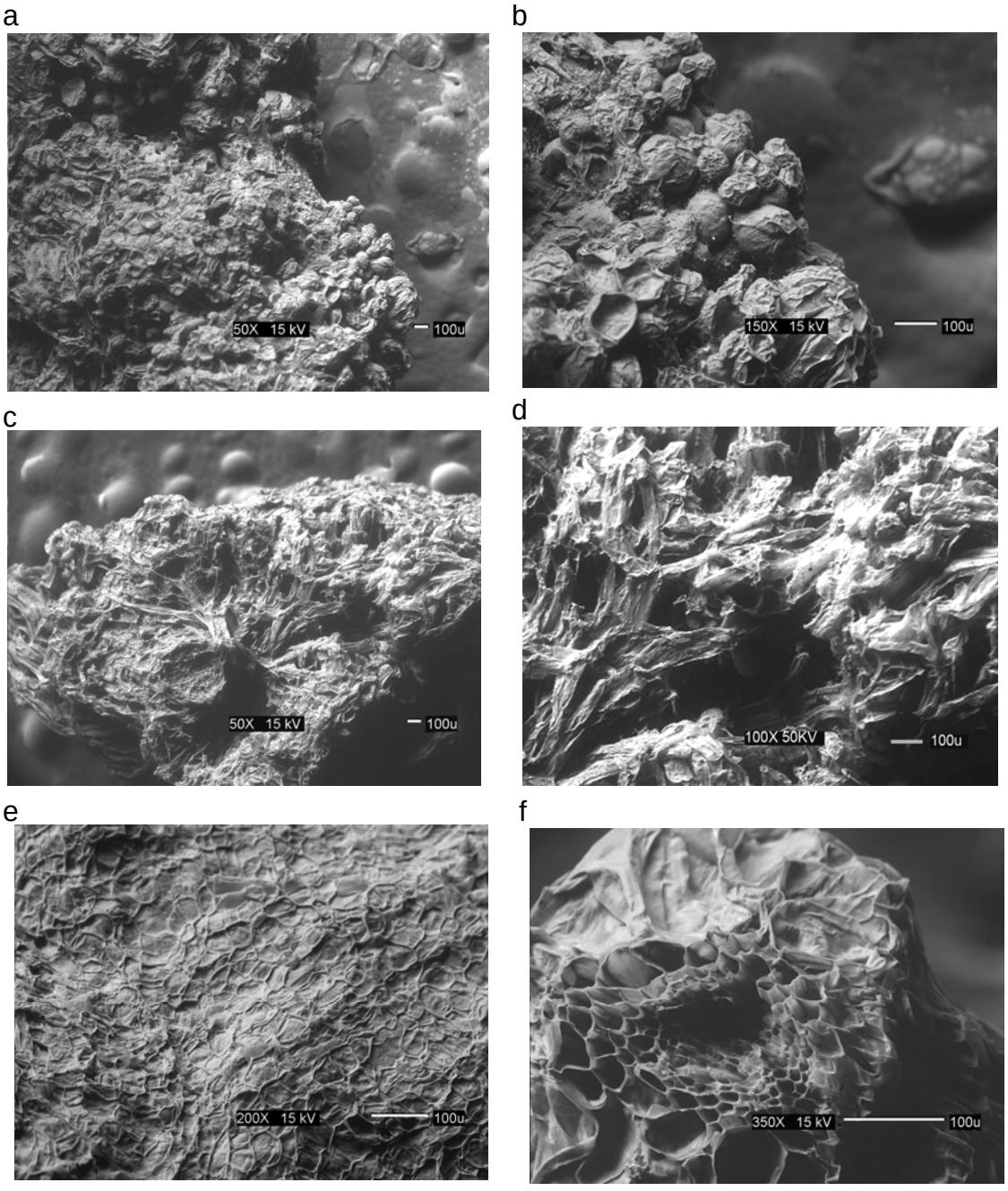

Figure 4.19. Calli samples analyzed by low vacuum scanning electron microscope. a) and b) Globose cells before collapse. Note the compact arrangement of the cells. c) and d) Filiform cells with a loose arrangement. Even though they are completely collapsed, their general aspect and arrangement can be discerned. e) Compact arrangement of superficial cells of the callus. d) A root-like structure emerging from a callus. Note the arrangement of the cells in this structure, which is reminiscent of the anatomy of a root.

5. DISCUSSION

5.1 Selection of Type of Explant and Possible Callogenic and Embryogenic Lines from 17 F3 Interspecific Bean Hybrid Lines

5.1.1 Induction of Callogenesis with 10mg/L of 2,4-D and Embryogenesis with 2,4-D and BA

In the initial phase, induction of callogenesis was tested for five different types of explants from 17 F3 lines. The calli produced from basal portions of cotyledons, epicotyls, radicles and hypocotyls using 10mg/L of 2,4-D varied from friable to nodular, and colorations included whitish, yellowish, greenish and brownish. The variation obtained may be indicative of the formation of calli with different morphogenic competence, since George (1996) has affirmed that embryogenic calli tend to have a nodular appearance. Indeed, most of the calli obtained in our experiments was of the nodular type and had a yellowish, greenish or brownish color, which according to George (1996), are the most amenable to the induction of somatic embryogenesis. On the other hand, we did not find translucent globular structures in nodular calli, as those obtained by Saunders et al (1987) and Hoyos (1990) when using 10 to 30mg/L of 2,4-D for the induction of callogenesis in cotyledons and leaf discs from seedlings of common beans.

With 10mg/L of 2,4-D, explants from cotyledons (apical and basal portions) produced calli at the cut-zone which eventually covered up to a third of the explant surface; while calli produced from epicotyl, radicle and hypocotyl explants eventually covered almost the entire explant surface. It can be deduced that these tissues have different levels of competence, and therefore require different treatments to achieve the precise balance of plant growth regulators that will induce the callogenic response. Similar differences in responses of the explants was observed by Coelho da Silva (2002), who noticed that in order to promote proliferation of calli from soybean hypocotyls explants, it was necessary to add a higher concentration of 2,4-D (2mg/L) than that required to induce calli in cotyledons (1mg/L). In common bean cotyledons, a higher concentration of 2,4-D might be necessary to achieve similar levels of callogenesis to those observed in other types of explants under 10mg/L of 2,4-D.

The percentage of necrosis in cotyledons explants (apical and basal portions) was higher than in epicotyl, hypocotyl and radicle explants. Furthermore, necrosis in epicotyl, hypocotyl and radicle explants was eventually overcome and callus growth resumed, while in all our experiments, necrosis resulted in the death of the cotyledon explants. Necrosis in the explants could be the result of accumulation of phenolic substances in epidermal and subepidermal cells, according to Puigderrajols et al (2000), who observed necrosis in cotyledons of cork oak (*Quercus suber* L.). A positive correlation exists between the use of 2,4-

D and the accumulation of such phenolic compounds that leads to browning (George, 1996).

The nodular calli obtained were transferred to a lower concentration of 2,4-D (0.1mg/L) in the presence of a cytokinin (BA). This approach follows Martins and Sondahl's report (1984), who observed somatic embryos of common bean at early stages of development when embryogenic calli were transferred to a lower concentration of auxin (IAA) and cytokinin (Kinetin). George (1996) also suggests that the development of embryogenic calli is favored under the presence of a combination of auxin and cytokinin. However, calli obtained in these experiments did not become nodular, with the exception of calli from hypocotyl explants, which also produced globular structures. The difference in auxin levels (10 vs. 1mg/L of 2,4-D) did not seem to play a determining role in the morphogenetic capacity of the explants. These results are in agreement with Jiménez's suggestion (2001) that there are other as yet unidentified factors that determine the competence of the tissues to develop embryogenic cells (Jiménez, 2001).

Calli from hypocotyl explants developed globular structures and roots. As an isolated event, a possible embryo with a heart shape was observed, which later reversed into callus. Heart-shape structures in calli were not frequent, but could give an indication of the possible embryogenic potential of the calli obtained from hypocotyls.

5.1.2 Effect of 1mg/L of 2,4-D On Callogenesis and 0.1mg/L of 2,4-D, 1mg/L of BA and 60g/L of Maltose on Embryogenesis

This experiment was designed to induce embryogenic calli and the subsequent development of embryos using a lower concentration of 2,4-D (1mg/L), a medium with a combination of auxin and cytokinin (0.1mg/L of 2,4-D and 1mg/L of BA) and a final transfer to a medium supplemented with maltose to mature embryos (as suggested by Moon and Hidelbrand, 2003). The results from this experiment did not show differences in the production of calli and their appearance following their transfer to a medium with auxin and cytokinin, and subsequently to one with BA and maltose; however, this did have an effect on necrosis. Necrosis developed only in explants from cotyledons (both apical and basal portions) and calli derived from these explants. Once again, this suggests that this type of explant is more sensitive to 2,4-D than the epicotyls, radicles and hypocotyls explants.

When the calli produced with 1mg/L of 2,4-D were transferred to a medium with 0.1mg/L of 2,4-D and 1mg/L of BA, nodular calli developed in cotyledon explants, while in epicotyl, radicle and hypocotyl explants a high proliferation of friable calli was observed. Clearly, for *P. vulgaris* the transfer of the callus from a higher to a lower concentration of 2,4-D (1mg/L to 0.1mg/L) in combination with BA (1mg/L) does not improve the induction of embryogenesis. This contrasts with the study of Jimenez and Guevara (2001), cited by Jimenez (2001), who

reported that the accumulation of auxins and cytokinins in the medium promotes the development of embryos of *Citrus*. Jimenez (2001) stated that cytokinins may be important during the initial phases of somatic embryogenesis, but not during later stages of embryo development and maturation. Our results also contrast with the positive effect of maltose reported in embryogenesis of soybean (Fernando et al., 2002 and Champrane et al., 2002). These results do agree with the study of Ramakishnan et al (2005), who found that maltose did not enhance the embryogenic competence of callus of cowpea (*Vigna unguiculata*).

Most of the 17 F3 lines that were assayed for the proliferation of calli showed high callogenic capacity. However, two F3 lines, 133 and 155, showed higher proliferation of nodular calli with the formation of globular structures, which could be early stages of embryo development or abnormal embryos (Saunders et al., 1986 and Hoyos, 1990). For this reason, lines 133 and 155 were selected for all subsequent experiments to induce callogenesis and embryogenesis.

5.2 Callogenic and Embryogenic Response of the Two Selected Hybrid Lines

5.2.1 Callogenic effect of 2,4-D

Explants of the two selected lines were cultured in presence of 0.1mg/L of 2,4-D and 1mg/L of BA to study the possibility of one-step embryogenesis (induction of callogenesis and embryogenesis in a single medium). One-step embryogenesis has been achieved for other *Phaseolus* species, including *P. acutifolius, P. aureus and P. coccineus* (Malix and Saxena, 1992).

As in previous experiments, cotyledon explants revealed a high proportion of necrosis (more than 50%), and callus production was restricted to their cut-zones. On the other hand, necrosis in epicotyl, radicle and hypocotyl explants did occur, but it did not inhibit the production of calli in any of the treatments. Production of calli was observed even without 2,4-D in epicotyl, radicle and hypocotyl explants, thus suggesting that BA is one of the determinants of callogenesis. This is in agreement with the results of Genga y Allavena (1991), who studied cotyledons of *P. coccineus* under 1mg/L of BA.

The treatment with the highest concentration of 2,4-D (0.1mg/L) in presence of BA turned out to be the best treatment for the production of nodular calli. The most highly callogenic explants were the hypocotyls, followed by radicles, both of

which produced nodular calli. Epicotyl explants, on the other hand, produced the three types of callus (friable, nodular and compact), while cotyledon explants (apical and basal portions) eventually died. These observations demonstrate that the type of explant is probably one of the decisive factors for the successful establishment of embryogenic cultures (Gaj, 2004). The results obtained in this experiment are surprisingly different from those observed in experiments that were similar, except for a pre-treatment with 1.0mg/L 2,4-D (section 5.1.2), where all formed calli were friable. This discrepancy is in line with George's observation that in vitro cultures can be influenced growth regulators that were used during earlier stages of cultures (George, 1996).

Epicotyl explants developed calli at the base, while also elongating and producing lateral and adventitious buds. The high concentration of cytokinin (1mg/L of BA), with respect to the auxin (0.1mg/L of 2,4-D), appears to have broken apical dominance, thus allowing the development of axillary buds.

Because of its potential to proliferate nodular callus and globular structures with roots, the treatment with 0.1mg/L of 2,4-D and 1mg/L of BA was selected to proliferate and maintain nodular calli for use in the maturation assays. Structures similar to the globular structures seen in our experiments have been identified by Martins and Sondahl (1984), Saunders et al. (1986) and Hoyos (1990), as possible early stages of embryo development.

5.2.2 Callogenic effect of 2,4-D at 29°C

Explants were cultured under various concentrations of 2,4-D (0, 0.01, 0.05 and 0.1mg/L of 2,4-D) at 29°C, in order to evaluate the proliferation of calli under stress (high temperature). Stress conditions have been reported to have a positive effect on the induction and development of somatic embryos (Gaj, 2004). At 29°C, 0.1mg/L of 2,4-D favored the production of calli (although mainly friable) in epicotyl, radicle and hypocotyl explants. Hypocotyl and radicle explants were highly callogenic and they developed globular structures at 0.1mg/L of 2,4-D. However, embryos did not mature. Dejong (1992) has suggested that the lack of embryo maturation may be due to a restriction in the production of certain proteins, like the 32KD-endochitinase, that are crucial for the process. These results contrast with the positive effect of high temperature (35°C) in somatic embryogenesis of leaf explants of a *Chicorium* hybrid reported by Decout et al. (1994). Drawing from this report, it is possible that in beans the optimal temperature to induce embryogenesis is even higher than 29°C. In *Chicorium*, for example, temperatures ranging from 25 to 30°C did not favor somatic embryogenesis, while higher temperatures promoted the process.

The elongation, etiolation, and the development of long roots in epicotyl explants, as well as root development in hypocotyl and radicle explants, could be due to the darkness conditions. Similar phenomena under comparable conditions were also reported by George (1996).

5.3 Maturation of Nodular Callus

During the maturation stage, both zygotic and somatic embryos undergo various morphological and biochemical changes. These include the development of storage of organs (cotyledons) that expand concomitantly with the deposition of storage compounds, the repression of germination, and the acquisition of desiccation tolerance (Thomas, 1993 cited by Arnold et al., 2002). Taking this into consideration, different experiments to favor such developmental changes were carried out.

Macroscopically, the calli cultured under maturation regimes were very similar to the controls, both presenting globular structures. However, samples observed under the dissection microscope, revealed important differences. In the experimental cultures, we found evidence of heart and torpedo-shaped structures that suggest embryogenic potential of these calli, and a positive effect of the maturation treatments used. This seems relevant, since at present there is no reported evidence of an effective protocol for the maturation of somatic embryos of common bean.

5.3.1 Effect of Air-drying

When nodular calli were air-dried for 15 days and transferred to a medium with 2,4-D and BA, structures were seen that were similar to torpedo-stage

embryos, thus suggesting a positive effect of desiccation on the maturation of embryos. These results seem logical, since it is known that desiccation influences the formation of proteins associated with the development of storage products in sexual embryos (Kermode et al., 1986 cited by George, 1996). Similar results were obtained by Kim et al (2001) and Moon and Hidelbrand (2003) for soybean embryos.

Notwithstanding some positive results, most of the explants proliferated only nodular calli, suggesting that most of the calli cells were committed to continue on a callus-proliferation genetic program, while few were able to switch to an embryogenic pathway.

5.3.2 The Effect of Liquid Medium

When nodular calli was transferred to a liquid medium with 2,4-D and BA, structures were produced that were similar to globular and heart-stage embryos. In contrast with the superficial development of embryos observed in calli of several species, like *Arabidopsis*, these embryo-like structures were found *within* the calli. There are reported cases, as in *Ranunculus sp,* in which the development of embryos was also observed in deep-seated cells of the calli derived from floral buds (Konar and Nataraja, 1969 cited by Raghavan, 2004). Should this be the norm for the common bean, it could account for the limited

number of embryos or embryo-like structures seen throughout this and other investigations.

The development of embryos could be favored by the vigorous agitation, which may provide sufficient oxygen to developing embryos (George, 1996). Further development of potential embryos was not observed, except in one isolated case. The presence of 2,4-D (0.1mg/L) and BA (1mg/L) in the liquid medium permitted the development of embryo-like structures, possibly globular and heart stages. Martins and Sondahl (1984) obtained globular embryos of common bean in liquid cultures supplemented with 1mg/L of 2,4-D (5µM) and 2mg/L of Kinetin (10µM), while in the study of Saunders et al. (1986) embryos at globular stage were developed in presence of 30mg/L of 2,4-D. Our results could suggest that the liquid culture supplemented with 2,4-D and BA promoted the development of these embryo-like structures up to heart-stage. However, Arnold et al (2002) has affirmed that even though 2,4-D is effective in the promotion of embryogenic cultures, its further presence can repress the subsequent growth of the somatic embryos by blocking the expression of genes required for the transition to the heart stage.

5.3.3 The Effect of $AgNO_3$

$AgNO_3$ has been used to enhance the development of somatic embryos in numerous species, because of its inhibitory effect on the production of ethylene.

For example, $AgNO_3$ has been shown to enhance the formation of normal embryos and plant regeneration in a low regeneration capacity variety of soybean (Santos et al., 1997).

An experiment to promote the maturation of globular embryos was made using nodular calli obtained with 0.1mg/L of 2,4-D and 1mg/L of BA, subsequently cultured with or without $AgNO_3$. Both, experimental calli and controls revealed globular structures that developed roots, and that may represent abnormal globular embryos. In one instance, under $AgNO_3$ treatment a structure with acute tips, which possibly represent abnormal shoot and radical apices, also developed (Figure 4.9). This may be an abnormal embryo without any apparent cotyledons or cotyledon-like structures. Aberrant embryo development may be associated with abnormalities in its embryogenic program, as suggested by Tomaz et al. (2001) in their studies of somatic embryos of two lines of *Citrus.*

Calli treated with $AgNO_3$ showed structures resembling globular and heart-stage embryos within nodular calli, similar to those obtained in liquid medium. Therefore, it is apparent that both, $AgNO_3$ and liquid medium, can promote development of embryos or embryo-like structures, but only up to a certain stage. It may be that $AgNO_3$ blocks the production of ethylene to a degree that allows the development of embryo-like structures at the heart stage. Meskaovi et al. (2000) observed that the reduction of ethylene was beneficial to the maturation of

somatic embryos of white spruce. On the other hand, Hays et al. (2000) suggest a favorable role of the ethylene in the expansion of cotyledons in somatic embryos of *Brassica napus*; so it may be that further development of the possible embryos of common bean requires an increase of ethylene.

5.3.4 The Effect of Desiccation in Combination with $AgNO_3$

The combination of air-drying for 15 days followed by transfer to a medium with $AgNO_3$ promoted the formation of embryo-like structures, similar to those that developed in air-dried calli without $AgNO_3$. This, along with the results from the previous experiment (culture in $AgNO_3$ without air-drying), suggests that in the common bean, desiccation does not enhance the embryogenic response to $AgNO_3$. Silver nitrate combined with partial desiccation did enhance the maturation of embryos and plant regeneration in barley (*Hordeum vulgare* L.) (Castillo et al., 1998), thus highlighting the potential complexity and species-specificity of embryogenic agents.

5.3.5 The Effect of Liquid Medium and $AgNO_3$

In liquid medium with $AgNO_3$, nodular calli developed, which contained structures resembling globular and heart-stage embryos. However, these structures were obtained in liquid medium regardless of the presence or absence of $AgNO_3$, again suggesting that, either the accumulation of ethylene in liquid

cultures of the common bean was not hindering embryo development, or in these experiments $AgNO_3$ did not effectively inhibit ethylene production. The agitation of the flasks, as well as the way in which they were covered with aluminum foil, possibly permitted enough oxygenation and gas exchange in the cultures, to render the accumulation of ethylene negligible, as it is affirmed by George (1996). In solid medium with $AgNO_3$ only globular structures with roots were formed.

The results obtained in all maturation experiments of nodular callus previously described, show a positive effect of desiccation, liquid medium and $AgNO_3$, either alone or in combination, resulting in the development of heart and torpedo-like structures. However, none of these experiments resulted in the development of cotyledonary embryos that develop into plants. In contrast, Moon and Hidelbrand (2003), who worked with soybean, accomplished plant regeneration through embryo dessication. Similarly, Kumar (1998) reported beneficial effects of $AgNO_3$ in the successful regeneration of numerous species through somatic embryogenesis.

5.4 Organogenesis

Buds obtained from calli developed on the basal zones of epicotyls, and lateral and apical buds from epicotyls cultured in 0.1mg/L of 2,4-D and 1mg/L of BA, were transferred to a medium with 0.5mg/L of BA to induce proliferation.

However, no proliferation was observed and necrosis ensued. These results contrast with those of several authors, including McClean and Grafton (1989), and Mohamed et al. (1992), and Santalla et al. (1998), who reported the formation of multiple buds and the elongation of shoots from cotyledonary node explants of common beans cultured in even higher concentrations of BA (1mg/L). On the other hand, when Veltcheva and Svetleva (2005) used a similar concentration of BA to that used in our experiments (0.5mg/L) as a precultivation treatment, pedicels explants from seedlings of common bean produced the highest numbers of adventitious shoots.

In contrast, when the buds were transferred to a medium with a higher concentration of BA (2.25mg/L) in the presence of $AgNO_3$ (1.7mg/L), multiple buds proliferated, and necrosis of the tissues was reduced. A possible interpretation is that when buds are treated with BA alone (0.5mg/L), ethylene has a negative effect on bud proliferation. Consequently, $AgNO_3$ favors organogenic process of common bean due to its inhibitory effect on ethylene synthesis. This has also been suggested by the studies of Carvalho et al. (2000) and Guidolin (2003). A reduction in the extent of necrosis by $AgNO_3$ was also confirmed by Guidolin (2003).

Regeneration and elongation of shoots were obtained when buds were transferred to a medium supplemented with 1µM BA (2.25mg/L), 10µM $AgNO_3$

(1.7mg/L) and 3µM GA_3 (1.5 mg/L). This result confirms the beneficial effect of this combination of regulators reported by Carvalho et al (2002).

To promote rooting, shoots were transferred to media with different combinations of BA, GA_3, NAA and $AgNO_3$; however, all these treatments failed to induce and proliferate roots. George (1996) has stated that the behavior of in vitro cultures at any given time can be influenced not only by growth regulators present in the medium at that moment, but also by regulators that were used during earlier stages of culture. Accordingly, it is possible that the failure to proliferate roots is due to the high concentration of cytokinin (2.25mg/L of BA) applied (and therefore accumulated by the tissues) during both, the proliferation and the elongation stages. The presence of NAA alone, or in combination with BA and GA_3 failed to induce root formation, which markedly disagrees with the high percentages of rooting reported by Kartha et al. (1981), Mohamed et al. (1992b), Carvalho et al. (2000), and Guidolin (2003) for shoots of common bean. Guidolin (2003) has suggested that root formation may require more extensive periods of culture. However, in our experiments not only root induction did not occur after 60 days of culture in rooting medium, but also a progressive necrosis was noticed in all the cultures. As George (1996) has pointed out, rooting requires the attainment of the appropriate balance of plant growth regulators to stimulate both, rhizogenesis and caulogenesis. Unfortunately, none of the combinations of regulators assayed achieved such balance.

In summary, the positive influence of the $AgNO_3$ in the survival and proliferation of buds was corroborated, even though its presence did not stimulate the formation of roots as it did in previous studies of common bean (Carvalho et al 2000), sugar beet (*Beta vulgaris* L.) (Gürel and Gürel, 1988) and cotton (*Gossypium hirsutum* L.) (Ouma et al., 2004).

5.5 Scanning Electron Microscopy

All tested fixation protocols, including chemical fixation, rapid freezing, slow freezing in a freeze drying apparatus, and slow cooling in cold ethanol, failed to preserve the morphology of *P. vulgaris* calli. However, turgent cells were observed in some treatments, allowing us to make inferences about the structure of these calli.

Glutaraldehyde and paraformaldehyde, have been extensively used as chemical fixatives to preserve a wide variety of tissues, since they can cross-link the molecules that form the specimen (Ruzin, 1999). Our results showed that after chemical fixation with these two chemicals, only a few cells appeared well preserved; therefore, the standard mixture of glutaraldehyde and paraformaldehyde – effective for the preservation of so many other plant tissues - is not adequate for common bean calli. Interestingly, using a similar chemical fixation protocol, Fernando et al. (2002), obtained optimal preservation of somatic

embryos developed from cotyledonary leaves of soybean. It is possible that distortions are not the result of inadequate fixation, but rather evidence of damage caused during subsequent steps of specimen preparation, such as dehydration and critical point drying (Goldstein et al. 2003). Goldstein et al. (2003) consider freeze-drying as an alternative to avoid the artifacts generated by chemical fixation, dehydration and critical point drying.

The use of cryo-protecting agents, such as DMSO, is considered useful in preventing ice-damage to the specimen (Reinhoud et al. 2000). However, our results clearly indicate that the use of this agent prior to cryofixation does not enhance the preservation of *P. vulgaris* calli. Cell collapse occurs to a greater extent when DMSO is used as a pretreatment. This also contrasts with the report of Goldstein et al. (2003), who sustain that this cryoprotectant enhances cryofixation of biological samples. The collapse of the cells in *P. vulgaris* calli could be indicative of dehydration caused by a hypertonic medium. A reasonable follow-up derived from this observation (albeit beyond the scope of this thesis) would be to conduct similar experiments with increasingly lower concentrations of DMSO, to determine which concentration -if any- affords protection against ice crystal damage, without adverse osmotic effects. Another possible interpretation of our results is that DMSO - or the concentration used - is particularly toxic to the *P. vulgaris* callus cells, as suggested by Reinhoud et al. (2000).

For calli cryofixed by rapid freezing, results were similar whether the cryogen used was liquid propane or liquid nitrogen. In both cases, most of the cells of the calli became collapsed and distorted. These results contrast with the beneficial role of cryogens (such as liquid propane) in the preservation of the morphology of many plant cells to be analyzed by light microscopy (Baskin et al. 1996) or scanning electron microscopy (Kaeser et al. 1989). It is possible that the distortion of *P. vulgaris* cells is due to the formation of large ice crystals during freezing, since Kaeser et al. (1989) have reported that cytoplasmic components of the cells of sorghum roots were lost by the formation of large ice crystals when the samples were cryofixed by immersion in liquid propane or nitrogen slush. Although this type of damage is commonly reported in studies of *transmission* electron microscopy (such as Kaeser's report), it does seem surprising that ice damage would even disrupt the external morphology of cells. Clearly, the uniqueness of *P.vulgaris* callus cells manifests itself not only in their dissension to commit to embryogenic processes, but even in the way their structure responds to common (and usually effective) fixation protocols.

Results of the slow freezing treatments (made by direct transfer of fresh calli of *P. vulgaris* to the freeze drying apparatus) were inconsistent. Although in preliminary experiments this protocol resulted in the preservation of the greater number of cells, when repetitions were made, preservation was not better than that accomplished through rapid freezing. The fact that an optimized fixation was not obtained consistently points to the potential complexity of interactions

between factors that influence the effectiveness of fixation, not the least of which may be the structural heterogeneity of *P. vulgaris* calli.

A fixation protocol similar to that used by Martinez-Montero et al. (2000) for the cryo-preservation of sugarcane callus, was assayed to determine its adaptability to preservation of calli cells of common bean. Slow cooling was achieved transferring the samples to cold ethanol (-80°C), followed by immersion in liquid nitrogen. The rationale behind such procedure is that vitrification of the water in the cells of the calli and in their surrounding environment occurs when samples are placed in the cold ethanol bath, hence avoiding the formation of large ice crystals that could affect the external morphology of some cells (Fujikawa and Jitsuyama et al., 2000). Nevertheless, of the cryofixation protocols assayed in this thesis, this was the least effective in preserving the morphology of *P. vulgaris* calli; all cells collapsed. It is known that the slower the rate of freezing, the larger the size of the ice crystals formed (Watt, 1997). So again, this was possibly the result of freezing injuries to the plasma membrane of calli cells, inflicted by large ice crystals formed during the slow cooling and freezing processes. This type of injury has been observed by Fujikawa and Jitsuyama (2000) in embryogenic cells of asparagus slow cooled at -40°C.

In contrast to the chemically fixed calli, all cryo-fixed calli presented a flattened surface layer or secretion that covered each callus at least in part. It is likely that this *is* part of the normal structure of the callus, and that it is removed

or not preserved by the chemical fixatives. A similar observation was reported for embryos of *Chicorium* sp. developed from cortical cells of root explants (Chapman et al. 2000); the embryos were said to be covered by a layer which Chapman et al. called *snt*. I hypothesize that in common bean calli this layer is formed or secreted by the surface cells to isolate the calli from the environmental conditions (culture medium).

Evidently, none of the fixation protocols assayed in this thesis is completely effective for the preservation of calli cells of *P. vulgaris*. At best, some of the cryofixation protocols may be adequate for the preservation of *some* superficial cells and for the elucidation of coarse characteristics of the callus. Structural and physiological characteristics, such as cell dimensions, thickness of cell walls, extent of vacuolation, and even presence of different metabolites within the cell cytoplasm, may affect the rate of freezing and therefore the effectiveness of the cryofixation protocol.(Kaeser et al., 1989). Thus, callus in general may be more difficult of cryo-fix than other plant tissues because of its cellular heterogeneity.

Moreover, even if all intrinsic characteristics of a particular type of tissue favor its adequate fixation by means of freezing, there are external factors that may hinder rapid freezing, thus rendering the protocol ineffective. One of this factors (in the case of rapid freezing) is the rate at which the tissues are plunged into the cryogen, which -when done by hand (without the use of specialized cryofixation equipment)- may be difficult to reproduce. This consideration may

account for the inconsistency in our results. Therefore, the potential use of cryofixation for the effective preservation of *P. vulgaris* calli should not be dismissed. Cryofixation of common bean calli may require the use of specialized mechanisms, as those suggested by Gilkey and Staehelin (1986) and Kaeser et al. (1989). Alternatively, chemical fixation may be done using more powerful fixatives, such as osmium tetroxide (OsO_4) (Chapman et al., 2000).

Since low vacuum microscopy permits the observation of un-processed samples (Goldstein et al. 2003), we used this technique to obtain a point of reference that would help us to decide which fixation protocol preserved the calli in a state that is closest to its natural state. However, under the low vacuum SEM, the collapse of the samples was almost instantaneous, and at best we had an opportunity to observe and photograph only a small part of the sample. The high voltage of the electron beam (10 and 15KV), combined with the degree of vacuum required for image formation in the SEM (350µbar), inevitably caused immediate collapse of the samples. Even when a lower vacuum condition was used (pressures above 350µbar) it was not possible to prevent collapse of the samples.

6. CONCLUSIONS

1. Out of the 17 F3 hybrid lines of *Phaseolus vulgaris* and *P. coccineus,* cultured under concentrations of 1mg/L or 10mg/L of 2,4-D, lines 133 and 155 exhibited highest proliferation of nodular calli with embryogenic potential.

2. Hypocotyls of seedlings, followed by radicle and epicotyl explants, were the best type of explant to produce nodular calli, while cotyledon explants (both, apical and basal) failed to proliferate nodular calli under similar culture conditions.

3. Necrosis was a limiting factor to the survival of cotyledonary explants. In contrast, necrosis in epicotyl, radicle and hypocotyl explants did not affect their survival, and hence their callogenic potential.

4. Concentrations of 10 and 1mg/L of 2,4-D, and the combination of 0.1mg/L of 2,4-D with 1mg/L of BA induced effectively callogenesis in epicotyl, radicle and hypocotyl explants and, at lower frequency, in cotyledonary explants (apical and basal portions).

5. The transfer of calli to a lower concentration of 2,4-D (1mg/L), or to a combination of auxin and cytokinin (0.1mg/L of 2,4-D and 1mg/L of BA), or to maltose, was not effective as a treatment for the induction of embryogenic processes in common bean calli.

6. The highest proliferation of nodular calli with embryogenic potential was achieved in a culture medium supplemented with 0.1mg/L of 2,4-D and 1mg/L of BA. Lower concentrations of 2,4-D (0, 0.01 and 0.05mg/L) resulted in decrease of production of nodular calli.

7. At 29°C, concentrations under 0.05mg/L of 2,4-D failed to induce callogenesis, while concentrations of 0.05 and 0.1mg/ L of 2,4-D permitted the development of friable calli mainly of epicotyl, radicle and hypocotyl explants. Under these conditions, radicle and hypocotyl explants developed globular structures, which did not further develop.

8. Embryo-like structures somewhat resembling normal embryos at the globular, heart and torpedo stage of development, were obtained when embryogenic calli were air-dried for 15 days, transferred to liquid medium under agitation (150rpm) or cultured with $AgNO_3$. Globular structures with roots, which may be abnormal embryos, were also produced in these treatments. Combinations of these factors did not enhance the

embryogenic effect observed when each treatments was applied individually.

9. This is the first report of globular and heart shaped embryo-like structures developing *within* calli of the common bean. Such internal location of development could account for the limited number of somatic embryos reported for this species.

10. While $AgNO_3$, applied either alone or in combination with desiccation and transfer to liquid medium, was not conducive to the formation of cotyledonary embryos capable of regenerating whole plants, its presence played an important role in the proliferation of buds and elongation of shoots. It did not, however, promote the development of roots.

11. The presence of 0.5mg/L of BA alone, did not permit the proliferation of buds of common bean; in contrast, it promoted necrosis. The combination of 2.25mg/L of BA and 1.7mg/L of $AgNO_3$ permitted the proliferation of buds.

12. The elongation of shoots was achieved with a combination of 2.25mg/L of BA, 1.7mg/L of $AgNO_3$, and 1.5mg/L of GA_3.

13. The rooting of the shoots produced via organogenesis was not favored by any of the combinations of BA, GA_3, NAA and $AgNO_3$ tested.

14. The use of the microscopy techniques (light and scanning electron microscopy) enabled the observation of embryo-like structures generally found *within* the calli.

15. Chemical fixation and cryofixation treatments failed to preserve the structure of common bean calli. However, several treatments allowed the observation of some turgent cells of different morphologies (either globose or elongated), which gives us an insight into the actual structure of common bean calli. In addition, cryofixation protocols revealed the presence of a flattened surface layer or secretion not detectable in chemically fixed calli.

16. Altough all tested fixation protocols failed to preserve the morphology of *P. vulgaris* calli, some observations could be made that allowed us to make inferences about their intrinsic structure. It is apparent that calli have a secretion or flattened layer that is not preserved when standard chemical fixations are applied. They may also include filiform loosely arranged cells, globose compactly arranged cells, or combinations of these two types of organization. These differences in cell morphology and arrangement could correspond to the different types of callus observed macroscopically (friable, nodular and compact).

7. RECOMMENDATIONS

A number of investigations have been conducted over the last three decades in order to establish effective protocols to promote embryogenesis and organogenesis in common bean, and although in recent years a few effective protocols to promote organogenesis in calli have been developed, this has not been the case for embryogenesis. In my attempts to advance knowledge in this area, I have come across a number of observations which, although beyond the scope of the thesis, merit further consideration. More extensive and in-depth studies would provide complementary information that would facilitate a fuller comprehension of somatic embryogenesis and organogenesis in common beans. Accordingly, I recommend the following as themes for future research in *P. vulgaris*:

- ✓ Histological analysis of the embryogenic process from nodular calli.
- ✓ Study of the effect of different liquid media and times of desiccation on embryo maturation
- ✓ Study of the production of ethylene in embryogenic and organogenic cultures of common bean.
- ✓ Evaluation of additional cryo-techniques for the study of callus morphology and its relation with embryogenic competence, by means of scanning electron microscopy.

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APPENDIX

Appendix 1. Statistical analysis for the callogenesis response of explants of 17 F3 hybrid lines in presence of 10mg/L of 2.4-D

Ho: There are no significant differences in the production of callus among the explant type cultured.

Ha: There are significant differences in the production of callus among the explant type cultured.

Variance analysis

Variable	N	R^2	R^2 Aj	CV
% explants with callus	85	0.49	0.34	22.40

F.V.	SC	gl	CM	F	p-value
Model	24757.76	20	1237.89	3.12	0.0003
explant	5972.07	4	1493.02	3.76	0.0083
Line (Bloque)	18785.69	16	1174.11	2.96	0.0011
Error	25425.13	64	397.27		
Total	50182.89	84			

Test:LSD Fisher Alfa:=0.05 DMS:=13.65743

Error: 397.2676 gl: 64

explant	Averages	n		
Apical portion of cotyledon	72.76	17	A	
Radicle	91.18	17		B
Epicotyl	91.18	17		B
Basal portion of cotyledon	92.65	17		B
Hypocotyl	97.06	17		B

Different letters indicate significant differences (p<= 0.05)

Conclusion: p<α: Ho rejected. There are significant differences among the production of callus regarding to the explant cultured

Appendix 2. Chi-square test for the callogenesis response of explants of 17 F3 hybrid lines in presence of 10mg/L of 2.4-D

Ho: The development of callus is independent of the explant

Ha: The development of callus depends of the explant culture

Contingency table

Absolute Frequencies
In columns:Callus

Explante	no	yes	Total
Apical portion of cotyledon	19	49	68
Basal portion of cotyledon	5	63	68
Epicotyl	6	62	68
Hypocotyl	6	62	68
Radicle	2	66	68
Total	38	302	340

Expected Frequencies
In columns:Callus

Explante	no	yes	Total
Apical portion of cotyledon	7.60	60.40	68.00
Basal portion of cotyledon	7.60	60.40	68.00
Epicotyl	7.60	60.40	68.00
Hypocotyl	7.60	60.40	68.00
Radicle	7.60	60.40	68.00
Total	38.00	302.00	340.00

Statistic	Value	gl	p
Chi square Pearson	25.66	4	<0.0001
Chi square MV-G2	22.62	4	0.0002
Conting. Coef. Cramer	0.19		
Conting. Coef. Pearson	0.26		

Conclusion: $p<\alpha$: Ho rejected. The development of callus depends of the explant cultured

Appendix 3. Statistical analysis for the callogenesis response of explants of 17 F3 hybrid lines in presence of 1mg/L 2,4-D.

Ho: There are no significant differences in the production of callus among the explant type cultured.

Ha: There are significant differences in the production of callus among the explant type cultured.

Variance analysis

Variable	N	R^2	R^2 Aj	CV
% explants with callus	85	0.82	0.77	21.04

F.V.	SC	gl	CM	F	p-value
Model	80808.82	20	4040.44	15.03	<0.0001
Line	1764.71	16	110.29	0.41	0.9749
explant	79044.12	4	19761.03	73.50	<0.0001
Error	17205.88	64	268.84		
Total	98014.71	84			

Test:LSD Fisher Alfa:=0.05 DMS:=11.23507

Error: 268.8419 gl: 64

explant	Average	n			
Apical part of cotyledon	19.12	17	A		
Basal Part of cotyledon	77.94	17		B	
Epicotyl	92.65	17			C
Hypocotyl	100.00	17			C
Radicle	100.00	17			C

Different letters indicate significant differences (p<= 0.05)

Conclusion: p<α: Ho rejected. There are significant differences among the production of callus regarding to the explant cultured

Appendix 4 Chi-square test for the selection of explants of 17 F3 hybrid lines hywith callogenic and embryogenic potential in presence 1mg/L 2,4-D.

Ho: The development of callus is independent of the explant

Ha: The development of callus depends of the explant culture

Contingency table

Absolute Frequencies
In columns: callus

Explant	no	yes	Total
Apical portion of cotyledon	54	14	68
Basal portion of cotyledon	15	53	68
Epicotyl	5	63	68
Hypocotyl	0	68	68
Radicle	0	68	68
Total	74	266	340

Expected Frequencies
In columns:Callus

Explant	no	yes	Total
Apical portion of cotyledon	14.80	53.20	68.00
Basal portion of cotyledon	14.80	53.20	68.00
Epicotyl	14.80	53.20	68.00
Hypocotyl	14.80	53.20	68.00
Radicle	14.80	53.20	68.00
Total	74.00	266.00	340.00

Statistic	Value	gl	p
Chi square Pearson	178.84	4	<0.0001
Chi square MV-G2	179.63	4	<0.0001
Conting. Coef. Crammer	0.51		
Conting. Coef. Pearson	0.59		

Conclusion: $p<\alpha$: Ho rejected. The development of callus depends of the explant cultured.