Ph.D. Thesis

Plant regeneration from sunflower protoplasts of two wild species *Helianthus giganteus* L. and *Helianthus maximiliani* Schrad. and cultivated sunflower *Helianthus annuus* L.

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CONTENTS

ABBREVIATIONS

BAP	6 - benzilaminopurin
NAA	1 - naphthaleneacetic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	gibberellic acid
B_1	thiamine-HCl
B_6	pyridoxine-HCl
V-KM	protoplast culture medium of Binding and Nehls
MS	Murashige and Skoog medium
B_5	Gamborg medium
WH	White medium
PABA	4 - aminobenzoic acid
MES	2 - (N-morpholino)ethanesulfonic acid

1. ABSTRACT

Methods of plant regeneration from protoplasts of two wild species (*H. giganteus* L. and *H. maximilliani* Schrad.) and a cultivated sunflower (*H. annuus* L. cv. Florom-328) are described. Leaf protoplasts isolated from *in vitro* grown plants of two wild species formed embryogenic colonies when cultured in agarose solidified droplets of modified V-KM medium and covered with liquid V-KM medium (both supplemented with NAA and BA). Protoplast derived colonies developed organized structures resembling somatic embryos. These structures could develop into normal and abnormal embryos when transferred to MS medium with different combinations of NAA and BA.Embryo germination with subsequent plant regeneration could be carried out on hormone free MS medium. Regenerated plants were successfully transplanted in soil. Protoplasts of cultivated sunflower were isolated from dark grown hypocotyls. They were cultured in droplets of agarose-solidified V-KM medium covered with liquid V-KM medium supplemented with different concentrations of NAA and BA. One week later colonies were subjected to 2,4-D for one week period. Further culture in V-KM with reduced concentrations of NAA and BA resulted in the appearance of somatic embryos. Maturation of embryos was achieved by culture them on MS medium supplemented with NAA, BA, GA_3 and $AgNO_3$. If embryos were transferred onto hormone free MS medium the frequency of shoot formation in the best case reached 9.6 percent of viable colonies (1.3 percent of protoplasts plated). Shoots with developed roots were transplanted into soil, others were grafted on hypocotyls of *in vivo* germinated seedlings. Eighty percent of grafted shoots and over 95 percent of rooted shoots survived. The plants flowered and produced 5 to 10 seeds each. Factors affecting the frequency of embryo formation and plant regeneration are discussed.

2. INTRODUCTION

2.1.Sunflower as an important world-wide crop

The genus *Helianthus* is placed in the tribe *Helianthea* of the family *Asteraceae* or *Compositae*. The basic chromosome number is n=17. All together 46 diploid, tetraploid and hexaploid species have been identified (Heiser et al.1969; Heiser,1978). Cultivated sunflower is a diploid, as are several wild species. Thirteen species are annuals, all diploids. The rest are perennials with chromosome number n=17, including *H.giganteus* and *H.maximiliani*, n=34 or n=51.

Sunflower is one of the most important oil crops in the world. It is grown mainly for its seeds that contain valuable oil. Now it ranks second among all oilseed crops in the world as a source of edible vegetable oil. There is also non-oilseed (confectionery) sunflower, which is lower in oil, but higher in protein. These seeds are utilized as a whole, alone or added to other food. Oilseed sunflower can be used for different purposes, besides vegetable oil, e.g. as a raw material for oleochemistry, and as a substitute for mineral oil in various applications. Sunflower has recently become a major crop of economic importance in the United States. Although sunflower has been important also in the USSR since the beginning of the twentieth century, its production increased in other countries only in the last 10 to 15 years. Major sunflower production areas are now in the USSR, United States, Argentina, China, Australia, France, Spain, Romania, Hungary, Yugoslavia, South Africa, and Canada. Additionally minor production areas occur in many countries of Central and South America, Africa, and Asia.

Sunflower cultivation is almost exclusively performed with hybrid varieties based on a common source of cytoplasmic male sterility (CMS), discovered by Leclercq (1969) in *Helianthus petiolaris* and transferred to *H.annuus* germplasm. Due to extensive usage of female lines with this cytoplasm for hybrid seed production, all hybrids cultivated worldwide are closely related - at least with regard to their cytoplasm. Consequently, the genetic basis of modern varieties is comparatively narrow. Therefore, any further expansion of production may lead into increasing problems due to epidemic diseases, since sunflower is very sensitive to various fungal pathogens, e.g.*Sclerotinia sclerotiorum*, *Plasmopara halstedii* (downy mildew) or *Puccinia helianthi* (rust). Breeders use a wide

range of germplasm resources in their breeding programs to broaden the genetic variability of sunflower. Wild species are reservoirs of valuable genes particularly for disease, pest, drought and frost resistance, CMS sources, wider environmental adaptation.

Genetic stocks of wild species undoubtedly play an important role in genetic improvement of crop plants including sunflower. But use of the wild species in sunflower breeding is limited by poor crossability and sterility in interspecific hybrids (Jan, et al.1988). Most attempts to obtain hybrids by sexual crosses between perennial diploid and annual species have failed. Interspecific hybrids have been obtained between *H.annuus* and some of the tetraploid and hexaploid perennials, particularly *H.tuberosus* (Heiser, 1978).

2.2. New methods of "biotechnology" in crop improvement

Intensive research in the last fifteen-twenty years in the field of plant cell and molecular biology have demonstrated great opportunities for the genetic manipulation of plants. Various cell culture techniques have been shown already to be relevant for numerous important crop species including sunflower, e.g. embryo culture (Alissa et al. 1985; Chandler and Beard, 1983; Espinasse et al. 1991; Kräuter et al.1991), meristem culture (Schrammeijer et al. 1990), anther and microspore culture (Witrzens et al.1988; Gürel et al. 1991), somatic hybridization based on protoplast fusion and cell culture, and molecular techniques including gene transfer (direct DNA uptake or transformation by infection with *Agrobacterium*; Everet et al. 1987; Schrammijer et al. 1990).

These and other methods can be very useful either in the production of identical plants or in increasing their variability. Entire plants can be propagated from different explants such as leaf sections, anthers, meristems, or even single isolated cells and protoplasts. Combining conventional crop improvement programs with new biotechnological methods could result in different useful applications, e.g. propagation *in vitro* (Binding and Krumbiegel-Schroeren, 1984; Thorpe and Patel, 1984), meristem culture for virus elimination (Kartha, 1984; Broome and Zimmerman, 1984), secondary product synthesis (Yamada, 1984; Whitaker and Hashimoto 1986), production of haploid plants (Bajaj,

1983), and obtaining new varieties through molecular genetic approach (Wullems et al. 1986).

These techniques are not only elegant demonstrations of model experimental systems, but they build up a new basis in agricultural biotechnology. New hybrids, mutants and genetically engineered plants with superior characteristics can be obtained and adapted for transfer into the breeding process. The following list quotes a few examples of recent, commercially developed, genetically engineered food crops (Erickson, 1992):

Asgrow Seed Company, Kalamazoo, Mich.

"Field-testing yellow squash, cucumbers and cantaloupes genetically vaccinated against four common plant viruses. Also developing genes for resistance to fungus and bacteria to protect these and other fresh-maker vegetables, including lettuce and carrots."

DNA Plant Technologies, Cinnaminson, N.J.

"Field-testing tomatoes that produce the enzyme chitinase, which repels fungi responsible for post-harvest rot, and tomatoes with an enzyme that extends vine ripening. Developing tomatoes with "antifreeze" genes from winter flounder. Also working with Du Pont on a genetically engineered canola that produces a high-temperature frying oil that is low in saturated fat."

Monsanto, St.Louis, Mo.

"By incorporating genes from soil bacteria, the company is developing a high-solids, low-moisture potato that will absorb less fat during deep frying and a tomato with delayed ripening. Also developing potatoes and tomatoes engineered to resist viral disease as well as potatoes protected against insects. Testing herbicide-resistant cottonseed for oil."

Pioneer Hi-Bred, Des Moines, Iowa.

"Testing sunflower and canola with altered amino acid profiles to make oils that are more nutritious and lower in saturated fat. Also developing high-methionine soybeans for live stock feed, corn resistant to insects and diseases, and alfalfa resistant to alfalfa mosaic virus."

However, there still exist serious problems in this rapidly expanding biotechnology industry. One of them is reliable plant regeneration from single cells or tissues that is required for stable integration of newly constructed genetical material into entire plants which then can reproduce and pass on the new traits to their progeny.

2.2.1. *Plant regeneration: the dominant problem in plant cell culture*

Sinnot (1960) has defined regeneration as "a tendency shown by a developing organism to restore any part of it which has been removed or physiologically isolated and thus to produce a complete whole". This general definition can be applied as well to the phenomenon of plant regeneration *in vitro*, since isolated tissues or single cells can be regenerated into whole plants under appropriate conditions.

Since Julius Sachs (1882) about a hundred years ago postulated the existence of specific substances as factors responsible for the initiation and development of organs in plants and Went (1938) in thirties confirmed his ideas, techniques of plant cell culture have added much to our understanding of the morphogenetic phenomena *in vitro*. However, this process of particular pattern of cell division with subsequent differentiation that leading to organ and eventually to plant formation is still largely unknown and our related knowledge is mostly empirical. However, this empirical knowledge allows us using proper plant regulators to initiate undifferentiated cell growth *in vitro* (callus or cell suspension cultures) in almost any species, but it remains unclear in many cases how to switch these cultures to the pathway of plant regeneration. There are some generalized principles and approaches (described below), based upon the experimental findings. The most critical factors found empirically affecting the morphogenesis *in vitro* are the followings: choice of genotype, type and physiological stage of explant, hormone concentration and sequence of application of different hormone combinations, mineral

salts in culture medium, nitrogen source, concentration of vitamins and other additives (amino acids, coconut water, casein hydrolysate, etc.), physical conditions such as temperature, quality, duration and intensity of light.

Morphological and histological studies on tissue culture of different species indicated that cells or tissues *in vitro* can produce plants through different pathways. These studies demonstrate that plants can be regenerated either by shoot formation followed by rooting them (organogenesis) or by embryo formation (somatic embryogenesis). It has been noted also that in both cases there are direct and indirect ways of regeneration. Direct regeneration in the absence of callus stage has been reported in *Oryza sativa* (Wernicke et al. 1981), *Sorghum* (Dunstan et al. 1978), *Dactylis glomerata* (Hanning and Conger, 1982) and some other species. Indirect regeneration after the production of callus from tissue or cells is, however, the most common pathway.

2.2.2. *Organogenesis*

Plant regeneration through organogenesis usually requires two main steps, i.e. induction of shoot formation and then rooting the shoots. The consequence is that at least two different media should be applied. As a rule, it takes repeated attempts and a rather long time to reach the stage when plants can be transferred into soil.

It has been shown by anatomical and histological investigations that shoots can be formed either directly from primary explant tissue or from intermediate callus. Shoots originate from groups of meristematically active cells (not from single cells as in the case of embryogenesis). These cell groups undergo several stages of differentiation that can lead either to tracheary element formation or to appearance of shoot primordia. The young shoot is connected *via* the vascular system to the original cells of the mother tissue. Multicellular origin of shoots obtained through organogenesis may cause problems in further genetic transformation experiments, because it increases the probability of obtaining chimaeric individuals.

As it has been indicated by Skoog and Miller (1957) a critical factor in controlling growth and morphogenesis is the concentration of growth regulators in the culture

medium. In general, a high concentration of auxin and a low concentration of cytokinin promote cell proliferation (formation of callus). If the auxin to cytokinin ratio is reversed, the result is usually induction of shoot formation. Auxin alone or in a combination with a low concentration of cytokinin is in many cases suitable for the induction of root development.

Besides callus culture to which the protocol described above can be applied there is another, as it is generally believed, a more suitable source of organogenic material - the cell suspension.

Steps corresponding to shoot and root induction are similar to that described above, except shoots are almost always regenerated on a solid medium. The efficiency of organogenesis can be increased if cell suspensions are subcultured a few times in regeneration medium with growth regulators, i.e. high cytokinin, then auxin on a solid medium with reduced auxin level.

Such advantage of liquid cell culture could be combined with callus culture procedure, i.e. initial incubation of explants in liquid regeneration medium with their subsequent transfer onto solid medium for shoot regeneration (Chraibi et al. 1992).

More and more crop plants get on in the list of species capable to undergo organogenesis. However, the plant regeneration occurred after initiation of organogenic culture can be utilized once, if the material in its regenerable stage can not be maintained for a long time. In many cases, regeneration becomes limited or even impossible after several years of culture maintenance. Because of this, regeneration from long-term cultures still remains to be problematic in many species.

2.2.3. *Somatic embryogenesis*

Somatic embryogenesis is a process of embryo formation from cells that are not produced by gametic fusion. Development of these "somatic" embryos is similar to that of natural zygotic embryos. Initiation and development of embryos from somatic tissues in plants has been first observed by Steward et al. (1958) and Reinert (1959) in *Daucus carota*. Since that time it has been observed in many other species. In some of them

somatic embryogenesis is the typical or exclusive way of plant regeneration *in vitro*.

In contrast to shoots regenerated through organogenesis, somatic embryos originate from single epidermal cells (Konar et al. 1972) or single cells presented in proembryonal masses (PEM) in callus or cell cultures (Vasil and Vasil 1981; Vasil and Vasil 1982). Development of somatic embryos starts with unequal division of the cell followed by differentiation into a bipolar structure that can be observed very well in cell suspension or protoplast cultures (Zaghmout and Torello 1989; Laine and David 1990; Tremouillaux-Guiller 1991; Dudits et al. 1991). Subsequent development shows globular, heart and torpedo stages also characteristic of zygotic embryo development. After maturation and germination of embryos the whole plant is formed.

Somatic embryos are often called embryoids. This term is usually applied to structures which resemble proembryos or embryos, but whose ability of further development into plants is not confirmed yet.

In *D.carota*, the most thoroughly investigated model plant for somatic embryogenesis, almost any part of the plant can be successfully used for somatic embryo production: immature embryos (Steward et al. 1964), hypocotyl (Fujimura and Komamine, 1979), young roots (Smith and Street, 1974), taproots (Steward et al. 1958), petioles (Halperin, 1966), peduncle (Halperin and Wetherell, 1964) and protoplasts (Kameya and Uchimiya, 1972). In many other species, however, only specific explants show any embryogenic response in culture. For most of these species immature zygotic embryos (Gamborg et al. 1970; Vasil and Vasil, 1980), young leaves (Haydu and Vasil, 1981), ovules (Litz and Conover, 1982) or immature inflorescences (Vasil and Vasil, 1981; Bretel et al. 1980) proved to be the best sources of embryogenic material.

The usual procedure of establishing actively dividing embryogenic callus or suspension cultures involves application of high levels of exogenous auxins (in most cases 2,4-D) for a certain period. These cultures usually consist of clusters of embryos at a very early stage of development - proembryogenic mass (PEM). When the concentration of auxin is reduced (or auxin is omitted from the medium altogether), PEM enter the "standard" pathway of embryo development (Lu and Vasil 1981; Wang and Vasil 1982).

2,4-D as a rather strong auxin can be applied in a very high concetration (up to 100 μM) for a short time, so called "auxin shock" (Dudits et al.1991). In some rare cases it was found that production of embryogenic cultures was mainly determined by physiological conditions of the tissue, independently of the growth regulators (Jelaska 1972; Pence et al. 1980). Maturation and germination of embryos are generally carried out on hormone free medium.

The regeneration of plants through somatic embryogenesis offers a number of advantages. In comparison to other pathway of plant regeneration - organogenesis - many more plants can be obtained by somatic embryogenesis. Plants can be produced in a relatively short periods of 3-6 weeks. Also it is possible to synchronize the development of somatic embryos that is very desirable for the establishment of automated systems and mass propagation. Because of the single cell origin of plants regenerated through development of somatic embryos this pathway of plant regeneration is would be preferable for use in genetic manipulation experiments.

Practical details of the above schemes of induction of embryogenesis will undoubtedly vary from species to species. However, there is a hope that in the near future - if the right genotype, explant and concentration of inducing auxins are selected - it will be possible to regenerate any species *via* somatic embryogenesis.

2.2.4. *Morphogenesis of sunflower in vitro culture*

Over the last ten years, a variety of techniques on plant regeneration of sunflower (*Helianthus annuus* L.) by organogenesis or somatic embryogenesis have been described. Several authors have reported shoot formation from different explants: hypocotyl (Paterson and Everett, 1985), immature embryos (Witrzens et al. 1988; Espinasse and Lay, 1989), stem pith (Sadu, 1974), cotyledons (Greco et al.1984; Power, 1987; Nataraja and Ganapathi, 1989; Knittel et al. 1991; Chraibi et al. 1991; Pugliesi et al. 1991; Chraibi et al. 1992; Ceriani et al. 1992). Using high concentration of sucrose Finer (1987) and Freyssinet and Freyssinet (1988) obtained direct somatic embryogenesis from immature zygotic embryos. Paterson and Everett (1985) and Wilcox (1988) could induce

indirect somatic embryogenesis from hypocotyls or immature embryos. Pelissier (1990) has developed a method to obtain somatic embryos from epidermis and parenchyma cell layers. Prado and Bervilie (1990) have induced somatic embryo development in suspension cultures of calli derived from hypocotyls of *H.annuus* and *H.petiolaris* but failed to obtain regenerated plants. Also successful applications of "embryo rescue" technique (Alissa et al. 1985; Chandler and Beard, 1983; Espinasse et al. 1991; Kräuter et al. 1991), meristem culture (Schrammeijer et al. 1990), anther and microspore culture (Witrzens et al.1988; Gürel et al. 1991) have been reported.

There are also several reports on plant regeneration of wild *Helianthus* species. Punia and Bohorova (1992) obtained plant regeneration through organogenesis and described morphogenetic ability of six wild species (*H.nuttallii* Tats., *H.mollis* Lam., *H.divaricatus* L., *H.debelis* Nutt., *H.maximiliani* S., *H.praexos* E & G) using different explants (stem and leaf pieces, cotyledons and buds). Krasnyanski et al. (1992) and Polgár and Krasnyanski (1992) established plant regeneration systems through somatic embryogenesis from leaf derived callus and suspension cultures of *H.giganteus* and *H.maximiliani.*

Although fertile plants have been regenerated from a variety of different sunflower tissues cultured, the protocols developed for plant regeneration often appear to be limited to particular genotypes. Furthermore, root development of regenerated plants occurs at rather low frequencies and prolongs culture time. This in turn results in the appearance of vitrificated plants which do not survive when transplanted to soil. Another problem is premature induction of flowering of shoots in culture that reduces seed yield in regenerated plants (Witrzens et al. 1988).

2.3. **Protoplasts as a tool in cell biology**

During the last two decades protoplasts have been extensively used as an experimental system for basic studies in plant cell biology. These experiments include investigations on cell organelles (Willmitzer, 1984; Lörz, 1984), cell wall regeneration (Fowke, 1980), membrane fusion (Willison et al.1982), virology (Takebe, 1984) and

membrane transport (Fowke and Constabel, 1985). Protoplasts were also used in studying fundamental cellular processes in plants as well as in the production of somatic cell hybrids by protoplast fusion (Constable, 1984), organelle transplantation (Wallin, 1984) and genetic transformation by direct DNA uptake (Ohyama, 1983).

2.3.1. *Plant regeneration from protoplasts*

Protoplasts can be easily isolated from a wide variety of plants using cell wall digesting enzymes. When protoplasts are cultured under specific conditions, they synthesize new cell walls and undergo cell divisions. The resulting callus cultures may be regenerated into plants (Giles, 1983; Vasil, 1984; Bajaj, 1989). Such ability of protoplasts to regenerate through cell culture into fertile plants is the main requirement of successful application of various established genetic manipulation techniques.

There are some obvious ways to attempt to solve current problem of plant regeneration from protoplasts: a) improving protoplast isolation techniques; b) screening of different media to determine the conditions inducive or permissive of expression of morphogenetic potential; c) searching for a new endogenous or synthetic phytohormones or anti-hormones; d) development of a new in a combination with previously established culture techniques.

Plant regeneration from protoplasts may occur *via* embryo or shoot formation. Embryogenesis from protoplasts was reported for the first time by Grambow et al. (1972) in carrot. Somatic embryos can be obtained from protoplasts indirectly *via* callus proliferation and differentiation into embryos (Pua, 1990; Saha and Sen, 1992) or directly from protoplasts into embryos (Song et al. 1990; Guang-min et al. 1992). Shoot formation usually results from protoplast derived calli due to differentiation of group of cells in callus tissue.

It appears that many factors contribute to the success of protoplast regeneration experiments. Some of the more important ones are discussed below.

a) Genotype and physiological state of donor tissue

Several authors have indicated very strict dependence of protoplast response to regeneration on genotype (Saha and Sen, 1992; Fischer et al. 1992; Burrus et al. 1991). Many genotypes used in different studies on protoplasts have been especially selected through tissue culture for their high regeneration ability (e.g. Pupilli et al, 1991; Guang-min et al, 1992).

Whether protoplasts are isolated from greenhouse grown plants, cell suspensions or *in vitro* maintained plants, the preisolation growth conditions and the age of plant material are very important for further experimental steps of protoplast culture (Watts et al. 1974; Shepard and Totten,1977; Kao and Michayluk, 1980).

b) Method of protoplast isolation

Since at present enzymes used for protoplast isolation are commercially available not much can be discussed about their purity and activity. The proper concentrations and compositions of enzymes can be determined in preliminary experiments.Most of enzyme mixtures used for protoplast isolation from different species contain cellulase (1-2%), macerozyme (0.1-0.5%), pectinase (0.5-2%) or pectolyase (0.1-0.5%). The pH of enzyme solution, time of incubation, light intensity, temperature, the concentration and type of osmoticum can affect protoplast yield and quality. In general, mannitol, sorbitol, glucose or sucrose are the major osmoticums (at the concentration of 0.4-0.6 M) used for adjustment of osmotic pressure in enzyme or washing solutions and in protoplast culture media (Chung 1988). It is important that the time of digestion should be kept to a minimum, since commercial enzyme preparations can contain nucleases, lipases and phosphatases (Potrykus and Shillito 1988; Gamborg et al. 1981) that may reduce protoplast viability at longer incubation time.

c) Composition of protoplast culture medium

It is impossible to give firm advice on how to choose the type of medium for a given protoplast culture. This is so because mineral salts, vitamins, osmotic and ionic agents, source of carbon, organic compounds, undefined nutrients such as casamino acids

and coconut water and growth regulators all can be important. A suitable strategy might be to start with a known medium found adequate in other systems (e.g. V-KM by Binding and Nehls, 1977; K_3 by Nagy and Maliga, 1976; L_4 by Lenee and Chupeau, 1989) and gradually modify it, since these media are based on cell culture media (MS of Murashige and Skoog 1962; B_5 of Gamborg et al, 1968; KM of Kao and Michayluk 1975).

Types and concentrations of growth regulators are the most variable parameters in protocols for different species. NAA and 2,4-D as auxins and BA, kinetin and zeatin as cytokinins proved to be the most effective growth regulators in protoplast culture media. The form of nitrogen source is also an important factor. Protoplasts of several species were shown to be sensitive to ammonium nitrate. Ammonium toxicity could be overcome in some cases by adding succinic acid (5-10 mM) to the medium .

d) Type and density of culture

Incubation in liquid medium is frequently used during the initial stages of protoplast culture. It is simple to carry out and subsequent handling of protoplast derived colonies is easy. In several cases, however, embedding in a supporting matrix (agar, agarose or alginate) was essential for the proper development of protoplast derived colonies. Agarose bead culture, i.e. culture of protoplasts in agarose blocks covered by liquid medium (Shillito et al. 1983) is very convenient and facilitates changing the composition of culture medium.

Initial protoplast density is not very critical as long as the minimum density required for division is maintained (this minimum is species dependent). It may, however, significantly influence subsequent behavior of protoplast derived colonies. This factor is rather important when protoplasts are embedded, because protoplast density cannot be changed as easily as in liquid cultures. If a low initial density is essential to the further development of colonies, the density can be increased to the minimum value by adding irradiated feeder cells (Perl et al. 1988) or by adding cells to the liquid phase of the culture ("nurse culture", Eigel and Koop, 1989).

It is apparent from the above discussion that research in the field of protoplast

regeneration is still empirical. Most workers in their studies on plant regeneration follow the question "How?" but not "Why?". This is rather understandable, since the basic processes which are involved in the plant regeneration are not exactly known. It is not clear yet, whether all living cells in plants are potentially able to form a complete new organism but we do not know yet how to permit their property to be expressed, or only limited number of cell types can carry over the morphogenic ability into cell culture. The last assumption seems to be more acceptable and could be even proved by numerous studies performed. If this is so, it makes more problematic to solve the problem of plant regeneration within next few years. Nevertheless, the number of plant species for which regeneration from protoplast is possible has increased during the past years and is over 280 (Roest and Gilissen, 1989).

2.4. Plant regeneration from protoplasts of *Helianthus* species

Data on plant regeneration from protoplasts in the genus *Helianthus* are rather limited. There have been several unsuccessful attempts at plant regeneration from protoplasts of wild species. Bohorova et al. (1986,1990) isolated protoplasts from leaves of axenic shoot culture of *H.praecox, H.scaberrimus, H.rigidus, H.nuttallii, H.mollis, H.divaricatus, H.debilis* and *H.maximiliani.* Protoplasts from all species were examined for division, colony and subsequent callus formation. No plant regeneration has been achieved. Recently, Chanabe et al. (1991) succeeded in regenerating plants from protoplasts of the wild species *H.petiolaris*. However, only a few shoots obtained from a single protoplast derived callus were successfully rooted and transferred to soil where they flowered.

Krasnyanski et al. (1992) and Polgár and Krasnyanski (1992) reported the regeneration of entire plants at high frequency through somatic embryogenesis from leaf mesophyll protoplasts of two wild species *H.giganteus* and *H.maximiliani.*

The first report on shoot regeneration from protoplast derived calli of cultivated sunflower has been published by Binding et al. (1981). Organization occurred in calli of shoot apex protoplasts. No information was presented concerning the reproducibility of

the system and whether it was possible or not to recover fertile plants. Subsequent attempts to develop plant regeneration systems for protoplasts isolated from various sources were mostly unsuccessful. Bohorova et al.(1986) isolated protoplasts from seedlings roots, hypocotyls and cotyledons of four cultivars. Only callus formation has been achieved. Lenee and Chupeau (1986, 1989) tested division capacity of protoplasts isolated from mesophyll, stems, cotyledons and hypocotyls. In this study they have also investigated nitrate and ammonium assimilation during protoplast development. Guilley and Hahne (1989) established conditions for donor plant culture, mesophyll protoplast isolation and their culture for regeneration of green, nodular, vigorously growing calli, but without plant regeneration. Moyne et al.(1988) and Dupuis et al.(1988,1990) described the early stages of protoplast differentiation into embryoids in a hypocotyl isolated protoplasts of sunflower (*H.annuus*). No further embryo development into plants was observed. Chanabe et al. (1989, 1991) examined the factors affecting the improvement of colony formation from hypocotyl, shoot and root protoplasts. Recently, Burrus et al. (1991) succeeded in obtaining organogenesis from protoplast derived calli of one particular genotype, a tissue culture selection of USDA germplasm release (CMS/*H.petiolaris Nutall*//CMS HA89 backcross). Two hundred-fifteen normal and abnormal shoots have developed on calli with 31 forming one or more roots. Fourteen plants with well developed root systems were transferred into the greenhouse. Viable seeds were recovered from 6 plants. However, the frequency of regeneration was very low (0.054 percent of protoplasts plated). The later report on a plant regeneration through organogenesis from protoplasts of cultivated sunflower published by Fisher et al. (1992) differs considerably from protocol of Burrus et al. (1991). The genotype used in this study was an inbred line. A characteristic element of their regeneration medium is its low concentration of growth regulators. Also, both hypocotyl and cotyledon protoplasts have been subjected to the identical treatment and exhibited comparable regeneration frequencies. However, the overall efficiencies in these studies are similar. While both protocols demonstrate the possibility of plant regeneration, the methods described are obviously not efficient enough for practical use.

The last study on plant regeneration from sunflower protoplasts has been reported by Krasnyanski and Menczel (1993). Using the commercial hybrid Florom-328 (identified as a genotype with high regeneration ability) plants were regenerated from hypocotyl protoplasts at comparatively high frequencies (1.3 percent of the original protoplast population plated). Regeneration has occurred through embryogenesis with subsequent shoot elongation in somatic embryos.

3. MATERIALS AND METHODS

3.1. *Helianthus giganteus*

3.1.1. *Plant material and protoplast isolation*

Plants of *H.giganteus* were obtained from the collection of Cereal Research Institute, Szeged, Hungary. They were maintained in the greenhouse in 20 cm pots in commercial mixed soil. Plants used for protoplast isolation were maintained under *in vitro* conditions.

Young leaves from one month old plants grown on plant maintenance medium (half-strength MS salts supplemented with 10 g l^{-1} sucrose) either with or without 0.2mg l^{-1} IAA were cut into 2 mm wide strips. Leaf pieces were digested in an enzyme mixture containing 1.0 percent cellulase "Onozuka" R10, 0.5 percent macerozyme R10 (Yakult Honsha Co.Ltd.,Japan) and 0.5 percent pectolyase (Seishin Pharmaceutical Co.Ltd.,Japan). The enzymes were dissolved in V-KM medium (Binding and Nehls 1977) and the pH was adjusted to 5.7. Digestion was carried out at 26°C in the dark for 20 hours (overnight). The resulting suspension was filtered through a nylon sieve, protoplasts were collected by centrifugation (50 x g, 2 minutes) and washed three times with a washing solution (16.0 g l^{-1} NaCl, 1.8 g l^{-1} $CaCl_2O$, 1.0 g l^{-1} glucose and 380 mg l^{-1} KCl). Purified protoplasts were resuspended in V-KM medium.

3.1.2. *Protoplast culture*

In all cases V-KM medium with 2.2 mg l^{-1} BA and either 0.01 or 0.1 mg l^{-1} NAA was used for the initial culture. Liquid cultures were initiated by resuspending protoplasts in V-KM medium at a density of 5 x 10^4 protoplasts per ml. Protoplasts also were cultured in agarose droplets covered with liquid medium (Shillito et al.1983). Double strength (0.5 percent) molten agarose (SERVA Feinbiochemica GmbH, Germany, cat.No.11404) was prepared in V-KM, cooled to about 40°C and mixed (1:1) with protoplasts. Final density of the mixture was 5 x 10^4 protoplasts per ml (in agarose droplet). Aliquots (1.0 ml) of the mixture were pipetted into 9 cm plastic Petri dishes.

After the agarose had solidified, the droplets were covered with 7.0 ml of liquid V-KM medium. The dishes were incubated in the dark at 26°C. Liquid medium was refreshed first time on the 7th day, then at 10 day intervals. Refreshing was carried out by replacing 5 ml of the covering liquid each time. Plating efficiency was calculated as the number of dividing cells (after ten days of culture) per number of protoplasts plated. Colonies about 1 to 2 mm in size (4 weeks after protoplast isolation) were released from the droplets by gentle pipetting in 2 ml of liquid V-KM. They were then transferred onto solid V-KM medium and the cultures were incubated in the dark at 26°C.

3.1.3. *Plant regeneration*

Protoplast-derived calli 4 to 5 mm in size were transferred to MS regeneration medium (Murashige and Skoog 1962) supplemented with 100 mg l^{-1} m-inositol, 0.1 mg l^{-1} $B_1^{vit.}$, 0.5 mg l^{-1} $B_6^{vit.}$, 0.5 mg l^{-1} nicotinic acid, 40 mg l^{-1} adenin sulfate, 50 mg l^{-1} glutamine, 30 g l^{-1} sucrose, 2.2 mg l^{-1} BA and 0.1 or 0.01 mg l^{-1} NAA (see Results). The cultures were incubated in the light (2000 lux light intensity, 16 hours photoperiod) at 26°C. Germination of mature embryos was carried out on hormone-free MS regeneration medium. Developing shoots were placed on plant maintenance medium.

3.2. *Helianthus maximiliani*

3.2.1. *Plant material and protoplast isolation*

H. maximiliani field grown plants were kindly provided by Dr. Gizella Németh in the Cereal Research Institute, Szeged, Hungary. They were transferred into commercial mixed soil and maintained in the greenhouse. Plants used as a protoplast source were maintained *in vitro* on half-strength MS medium supplemented with 10.0 g l^{-1} sucrose and 0.01 mg l^{-1} NAA.

Leaves from 3 week-old plants were cut into strips (2 mm wide) and incubated in an enzyme mixture containing 1.0 percent cellulase "Onozuka" R10, 0.5 percent

macerozyme R10 (both from Yakult Honsha Co. Ltd., Japan) and 0.1 percent pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan). The enzymes were dissolved in modified V-KM medium (80 g l^{-1} sorbitol instead mannitol, 20.0 g l^{-1} sucrose, 2.0 percent coconut water and 1.0 g l^{-1} glutamine, osmolarity 580 milliosmol, pH 5.6). Digestion was carried out at 25°C in the dark for 16-18 hours. The resulting suspension was filtered through a nylon sieve (100 μm pore diameter) and centrifugated (50 g x 2 min) to collect the protoplasts. Protoplasts were then washed three times in W58 solution (1.8 g l^{-1} $CaCl_2$ x $2H_2O$, 16.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl and 1.0 g l^{-1} glucose, pH 5.6).

3.2.2. *Protoplast culture*

Protoplasts were cultured in modified V-KM medium (see above) using the agarose droplet culture technique of Shillito et al. (1983). Molten agarose (Serva Feinbiochemica GmbH, Germany, cat. No. 11404) was prepared by autoclaving 0,5 percent agarose in V-KM medium. Protoplasts were resuspended in V-KM medium at a density of 8.0 x 10^4 ml^{-1} and were mixed (1:1) with molten agarose cooled to about 40°C. One ml of the mixture was placed in plastic Petri dishes (9 cm in diameter) and allowed to solidify. Finally the droplet was covered with 7.0 ml of liquid V-KM medium and the dishes were incubated in the dark at 25°C. Five ml of the covering liquid medium was refreshed every 8 to 10 days. Three weeks later initial hormones in liquid phase have been changed or remained the same (Table 2.). After six weeks of culture frequency of colony formation was determined. Subsequently the colonies (1 to 2 mm in size) were released from the agarose by gentle pipetting in 2.0 ml of V-KM medium, and they were transferred onto MS regeneration media supplemented with 100 mg l^{-1} m-inositol, 0.1 mg l^{-1} $B_1^{vit.}$, 0.5 mg l^{-1} $B_6^{vit.}$, 0.5 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 30 g l^{-1} sucrose (pH 5.8) and different concentrations of NAA and BA (Table 2). The cultures were incubated in the light (16 hours photoperiod, 1500 lux light intensity) at 25°C.

3.2.3. *Plant regeneration*

Embryos on different stages of their development with part of callus tissues were

transferred on hormone free MS regeneration media for further development. Rooted shoots were planted in soil or maintained under *in vitro* conditions.

3.3. *Helianthus annuus*

3.3.1. *Plant material*

Seeds of commercial hybrids were obtained from the Hungarian Seed Company, Budapest. The following hybrids were used: Barbara, Blumix, Citosol-3, Citosol-4, Clubsol, Florakisz, Florom-206, Florom-328, Florom-350, Fundulea-206, GK-Napszirom, Granchaco, J-550, Multibest. Seeds of inbred lines were kindly provided by Dr. László Takács (Feed Crop Research Institute, Iregszemcse, Hungary).

Germination of seeds: Seeds were surface sterilized by soaking in diluted (5-fold) commercial bleach for 20 minutes, washed three times in distilled water and kept overnight in distilled water at 4°C. Next day the cover of seeds was removed and the seeds were sterilized again by soaking in 70 % ethanol for one minute followed by soaking in diluted (5-fold) commercial bleach for ten minutes. They were then washed three times in sterile distilled water. For protoplast isolation seeds were germinated in the dark at 26°C on half-strength MS salts (Murashige & Skoog 1962) supplemented with 0.5 % sucrose and 0.6 % agar. Hypocotyls used in the screening for regeneration ability were germinated according to Paterson and Everett (1985).

Testing of regeneration ability: Hypocotyl segments were cultured and tested for the presence of embryos and shoots according to the method of Paterson and Everett (1985).

3.3.2. *Protoplast isolation*

Five day-old hypocotyls were cut into 5 to 10 mm sections and each section was cut in half longitudinally. One gram of cut hypocotyl tissue was placed in 10 ml of enzyme solution containing 2.0 % cellulase "Onozuka" R10 and 0.5 % macerozyme R10 (Yakult Honsha Co., Ltd., Japan). The enzymes were dissolved in the protoplast washing solution KS (18.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 6.13 g l^{-1} $CaCl_2$ x $2H_2O$, 3.3 mM MES, pH

(5.6). Digestion was carried out overnight (16 to 18 hours) at 26°C in the dark. Digested material was passed through nylon filters with 63 and 21 micron pore diameter, collected by centrifugation (80 g, 3 minutes) and washed twice in KS solution.

3.3.3. *Protoplast culture*

Purified protoplasts were resuspended in hormone free V-KM medium (Binding & Nehls 1977; with the following modifications: glucose, amino acids and nucleic acid bases omitted, 80.0 g l^{-1} sorbitol instead of mannitol, L-glutamine added at 1.0 g l^{-1}, osmolarity 0.58 osmol, pH 5.6) at a density twice as high as the desired final density in the agarose droplets. Aliquots (0.5 ml) were then mixed with equal volumes of V-KM medium containing different concentrations (Results and Discussion) of agarose (SERVA, cat. No. 11404) cooled to 40°C after autoclaving. The mixture was pipetted into 9 cm diameter plastic Petri dishes (GREINER, Germany). Solidified droplets were then covered with 10.0 ml of liquid V-KM medium supplemented with different amounts of NAA and BA (Results and Discussion). When a low density of protoplasts (5 x 10^3 ml^{-1}) was used inside the agarose droplets, the covering liquid medium also contained hypocotyl protoplasts at a density of 3 x 10^4 ml^{-1} ("nurse" cultures). The cultures were incubated in the dark at 26°C.

Induction of embryogenesis: One week later the liquid was replaced by 10.0 ml of V-KM with different hormones (Results and Discussion). After another week in the dark plating efficiency was determined. The liquid was then replaced by 10.0 ml of V-KM supplemented with 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} BAP (V-KM-3) and the cultures were incubated in the dark for another week. The agarose droplets were then dispersed by gentle pipetting in liquid V-KM-3 (10.0 ml). The resulting liquid cultures were diluted with V-KM-3 to give a density of about 100 colonies per ml and they were incubated for additional 4 days in the dark.

3.3.4. *Plant regeneration*

Colonies growing in liquid V-KM-3 were transferred onto regeneration medium

containing the basic salts of MS, B_5 or White media (Murashige & Skoog 1962; Gamborg et al. 1968; Shing & Krikorian 1981) supplemented with 100.0 mg l^{-1} m-inositol, 0.5 mg l^{-1} B_6^{vit}, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} B_1^{vit}, 1.0 mg l^{-1} $AgNO_3$, 0.2 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3, 0.01 mg l^{-1} NAA, 0.5 g l^{-1} enzymatic caseine hydrolyzate and 30.0 g l^{-1} sucrose, pH 5.8. Media were solidified by 0.3 % Phytagel from SIGMA. The cultures were incubated in the light (2000 lux light intensity, 16 hour photoperiod). After 8 to 10 days the developing somatic embryos were separated from the surrounding non-embryogenic calli and were transferred to fresh regeneration medium. Two weeks later the embryos were transferred onto hormone free MS salts supplemented with 100.0 mg l^{-1} m-inositol, 0.2 mg l^{-1} B_1^{vit}, 20.0 g l^{-1} sucrose and 0.26 % Phytagel (pH 5.8).

Transfer of plantlets into soil: Shoots 1 to 1.5 cm in length were separated from the surrounding callus material. Shoots with roots were directly placed into 10 cm pots containing sterilized commercial garden soil. The pots were covered by transparent plastic foil for a few days to prevent evaporation. Shoots without roots were cut about 5 to 8 mm below their tips and were grafted *in vivo* on hypocotyls of another commercial hybrid cultivar germinated in sterilized soil. Hypocotyls with the grafted shoots were covered with glass tubes for one week to prevent evaporation. Grafting was carried out as suggested by Dr. B. Pelissier (Rhone-Poulenc, Lyon, personal communication) with some modifications. All transplanted plants were incubated in an environmental chamber (CONVIRON, Canada; set to 24°C, 9000 lux light intensity, 12 hour photoperiod).

4. RESULTS AND DISCUSSION

4.1. *Helianthus giganteus*

Isolation and culture of leaf mesophyll protoplasts

Several different protocols of protoplast isolation were tried in preliminary experiments. Important factors were the composition of enzyme mixture, age and physiological state of the *in vitro* maintained plants. Plants that were maintained on MS medium supplemented with IAA yielded more protoplasts than those on hormone free MS. However, protoplast quality and initial division frequency were better in the second case. Due to incomplete digestion, no protoplasts could be obtained when plants older than 5 to 6 weeks were used. Even with young plants, use of pectolyase in the enzyme mixture was essential. Using conditions described in Materials and methods, 4 to 5 x 10^6 protoplasts could be obtained from each gram of leaf material collected from *in vitro* grown plants (Fig.1).

Initial culture of protoplasts was attempted in both liquid and agarose solidified V-KM media. First division occurred after 3 days in liquid and 6 days in solid medium. Initial plating efficiency was also different: about 60 percent in liquid and 12 to 15 percent in solid medium (dividing cells were counted 10 days after initiating the cultures). Efficiency of colony formation in agarose-solidified media was in range of 4.8 to 10.7 percent.

Further development of colonies resulted in very different morphologies in liquid and solid media. Colonies growing in liquid culture contained vacuolated, oval shaped cells which were only loosely attached to each other. They became frequently fragmented and from the fragments new colonies developed. In contrast, colonies in agarose solidified medium have consisted of spherical cells rich in cytoplasm. They appeared compact and globular in shape. Eventually, globular structures resembling somatic embryos developed on the surface of mother colonies (Fig.2A). While liquid media seem to give better plating

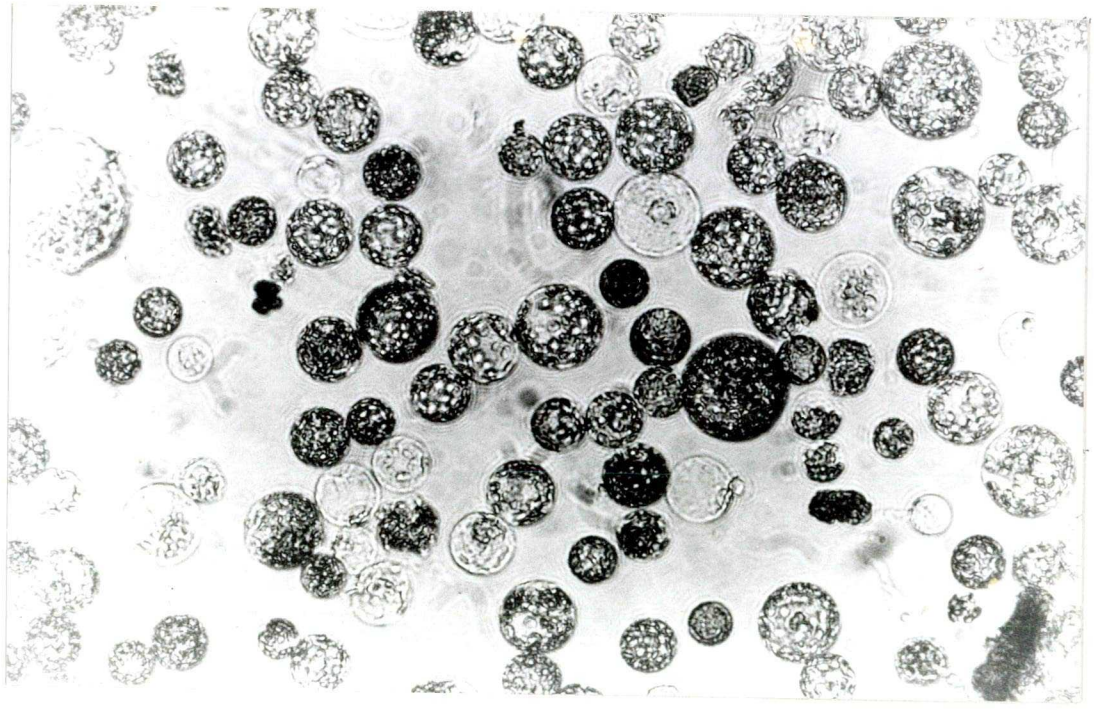

Figure 1. Freshly isolated leaf mesophyll protoplasts of *H.giganteus.*

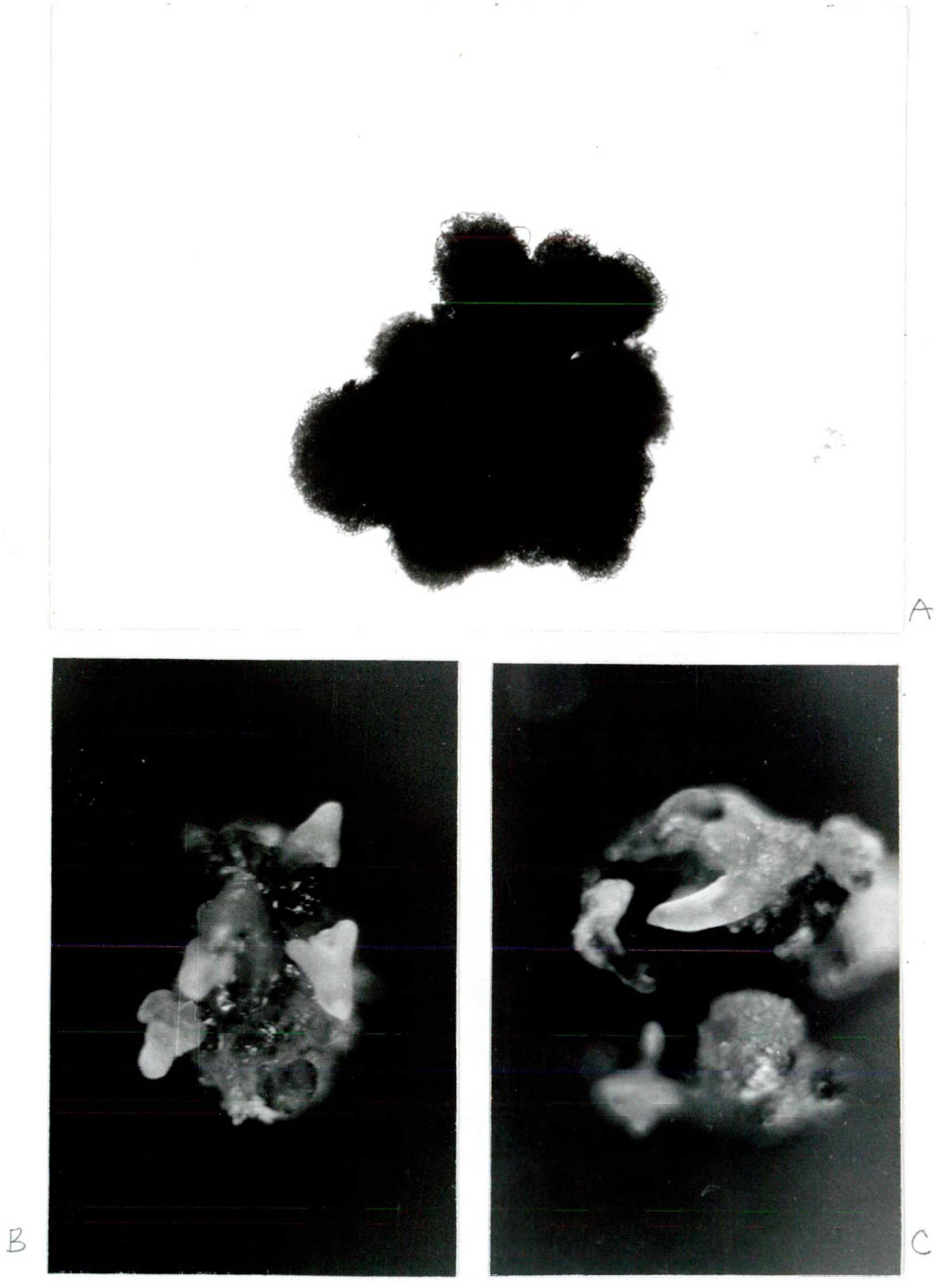

Figure 2. Regeneration of somatic embryos from *H.giganteus* protoplast derived calli. **A**, developing colony with attached embryoids; **B**, normal and **C**, abnormal somatic embryos developing on protoplast derived calli.

efficiencies, agarose droplet cultures appear superior with respect to the overall morphology and regeneration ability of colonies. This is in agreement with the results of Bohorova et al. (1986) who concluded that the use of agarose is essential in *Helianthus* protoplast culture.

Plant regeneration from protoplast derived embryogenic calli

Regeneration of plants was attempted using compact colonies obtained from agarose droplet cultures. They were released from agarose (see Material and methods) and were grown for two weeks on the solid V-KM medium supplemented with 2.2 mg l^{-1} BA and either 0.1 or 0.01 mg l^{-1} NAA. This treatment promoted fast callus growth and the formation and cell differentiation into organized embryo-like structures.

In order to promote maturation of embryos, calli were then placed on MS media supplemented with different hormone combinations. After two weeks of culture in the light, both normal and abnormal embryos could be observed on the calli (Fig.2B and 2C, respectively). At this point the proportion of calli showing embryo formation was determined (Table 1). Embryogenesis in calli initiated at low concentrations of NAA (0.01 mg l^{-1}) and subcultured using the same level of auxin was inferior to that of calli initiated and grown at higher auxin levels (0.1 mg l^{-1}).

Mature embryos were germinated on hormone free MS regeneration medium. Attempts to germinate single embryos (even with well developed cotyledons and roots) on this medium have failed in most cases. Vigorously growing plantlets were obtained only if clusters of embryos or embryos together with a small amount of callus tissue were transferred. Later these plantlets could be separated from the callus, rooted and maintained *in vitro* or in the greenhouse (Fig.3). Plantlets could be regenerated in every case when the original calli had shown embryogenesis. Plant regeneration, therefore, is practically identical to the frequency of embryogenesis shown in Table 1. Plantlets from altogether 20 embryogenic calli were rooted and transferred to the greenhouse.

The quality of source plants (whether maintained on IAA containing or hormone free MS media) does not seem to influence the frequency of embryogenesis. There is no data on the influence of genotype, since our original plants were all from the same

source. Embryogenesis could be induced reliably. Essentially the same results were obtained in three independent experiments.

Table 1. Influence of NAA concentration on frequency of embryogenesis in protoplast derived *H.giganteus* colonies.

NAA concentration in V-KM medium (mg l^{-1})*	NAA concentration in MS medium (mg l^{-1})	Efficiency of colony formation (%)	Frequency of embryogenesis (%)**
0.01	0.01	4.8	33
0.01	0.1	6.9	58
0.1	0.01	9.6	57
0.1	0.1	10.7	65

*All concentrations of NAA in V-KM and MS media were combined with 2.2 mg l^{-1} of BA.

**Frequency of embryogenesis indicates the percent of embryogenic calli of total number of calli obtained.

Figure 3. **A**, plants regenerated from *H.giganteus* protoplasts and maintained *in vitro*; **B**, regenerated plant transferred into soil in the greenhouse.

Summarized protocol of established plant regeneration system from leaf mesophyll protoplasts of *Helianthus giganteus* L.

a) embedding isolated leaf mesophyll protoplasts ($5x10^4$ prot/ml) in 1 ml of 0.25% agarose covered with 7-10 ml of liquid V-KM1 medium, culture them for 4 weeks with refreshing medium at 10 days interval, incubate in the dark at 26°C;

b) release of colonies (1-2 mm in size) and transfer them onto V-KM2 medium for 2 weeks, incubate in the dark at 26°C;

c) transfer of calli on MS1 medium for 2-3 weeks for embryo formation and maturation, incubate in the light (2000 lux light intensity, 16 hours photoperiod) at 26°C;

d) transfer of mature embryos on MS2 medium for germination;

e) transfer of shoots failed to develop roots on rooting medium;

f) transfer of plants with roots in soil, keeping them for the first 1-2 weeks under the glass cover or plastic foil (to prevent fast evaporation) or maintain in vitro on maintenance medium.

For procedure of protoplast isolation and embedding protoplasts in agarose and rooting medium composition that was the same as plant maintenance medium see Material and methods, for media used in protocol see next page.

Media composition for protoplast culture and plant regeneration from leaf mesophyll protoplasts of *Helianthus giganteus* L.

	V-KM1	V-KM2	V-KM3	MS1	MS2
Macronutrients	V-KM	V-KM	V-KM	MS	MS
Micronutrients	V-KM	V-KM	V-KM	MS	MS
Vitamins (mg/l)					
Biotin	0.1	0.1	0.1	-	-
i-Inositol	100	100	100	100	100
Nicotinic acid	1	1	1	0.5	0.5
Ca-panthotenate	1	1	1	-	-
Pyridoxin-HCl	1	1	1	0.5	0.5
Thiamin-HCl	10	10	10	1	1
Ascorbic acid	2	2	2	-	-
Cholin chloride	1	1	1	-	-
Folic acid	0.4	0.4	0.4	-	-
vit.A	0.01	0.01	0.01	-	-
vit.D_3	0.02	0.02	0.02	-	-
PABA	0.02	0.02	0.02	-	-
Organic constituents (g/l)					
Fructose	0.25	0.25	0.25	-	-
Sorbitol	0.25	0.25	0.25	-	-
Ribose	0.25	0.25	0.25	-	-
Xylose	0.25	0.25	0.25	-	-
Mannose	0.25	0.25	0.25	-	-
Rhamnose	0.25	0.25	0.25	-	-
Cellobiose	0.25	0.25	0.25	-	-
Na-Pyruvate	0.02	0.02	0.02	-	-
Fumaric acid	0.04	0.04	0.04	-	-
Citric acid	0.04	0.04	0.04	-	-
Malic acid	0.04	0.04	0.04	-	-
Casein hydrolysate	0.25	0.25	0.25	-	-
L-Glutamine	1.0	1.0	1.0	0.05	0.05
Adenine sulfate	-	-	-	0.04	0.04
Mannitol	80	80	-	-	-
Sucrose	20	20	20	20	20
Coconut water (ml/l)	20	20	20	-	-
Hormones (mg/l)					
NAA	0.1	0.1	0.1	0.1	-
BA	2.2	2.2	2.2	2.2	-
Others					
MES (mg/l)	640	640	-	-	-
Agar (g/l)	-	-	6.0	6.0	6.0
pH	5.6	5.6	5.8	5.8	5.8

4.2. *Helianthus maximiliani*

Isolation and culture of protoplasts

Mesophyll protoplasts were isolated from leaves of *in vitro* grown three weeks-old plants (Fig.4). From each gram of leaf material 2 to 3 million protoplasts could be obtained under the conditions described in Materials and methods. In our previous experiments on protoplasts of *Helianthus giganteus*, the V-KM medium of Binding and Nehls (1977) appeared superior to the others. For this reason we have not tested other protoplast culture media in this study. V-KM media with three different combinations of hormones were used for the initial culture of protoplasts (Table 2). Initial division rate and frequency of colony formation were both significantly influenced by culture conditions.

First divisions were observed after 5 to 6 days (after 8 days in cultures with the lowest hormone concentration). Plating efficiency was determined after two weeks. As shown in Table 2, the highest plating efficiency was found in the medium supplemented with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} BA. In order to promote further development of colonies, different hormone combinations (reduced, increased, or the same as the initial) were used in the liquid phase of cultures. The best yield of colonies was obtained using 1.0 mg l^{-1} of both NAA and BA for initial culture, followed by a reduction to 0.5 mg l^{-1} after three weeks (Table 2).

After four weeks two morphologically distinct types of colonies were found in the cultures. The first type consisted of relatively small cells rich in cytoplasm. They had roughly globular shapes resembling somatic embryoids (Fig.5A). The rest of the colonies contained larger, vacuolated cells and appeared more friable (Fig.5B).

Plant regeneration from protoplast derived calli

In order to initiate callus growth and embryo formation, colonies were removed from agarose and placed on MS media supplemented with the same hormones that were used in the V-KM media for colony growth. Development of embryos was observed after four weeks in culture . Both normal and abnormal embryos were found mixed within the same calli. At this point, frequency of embryogenesis in calli was determined. Essentially

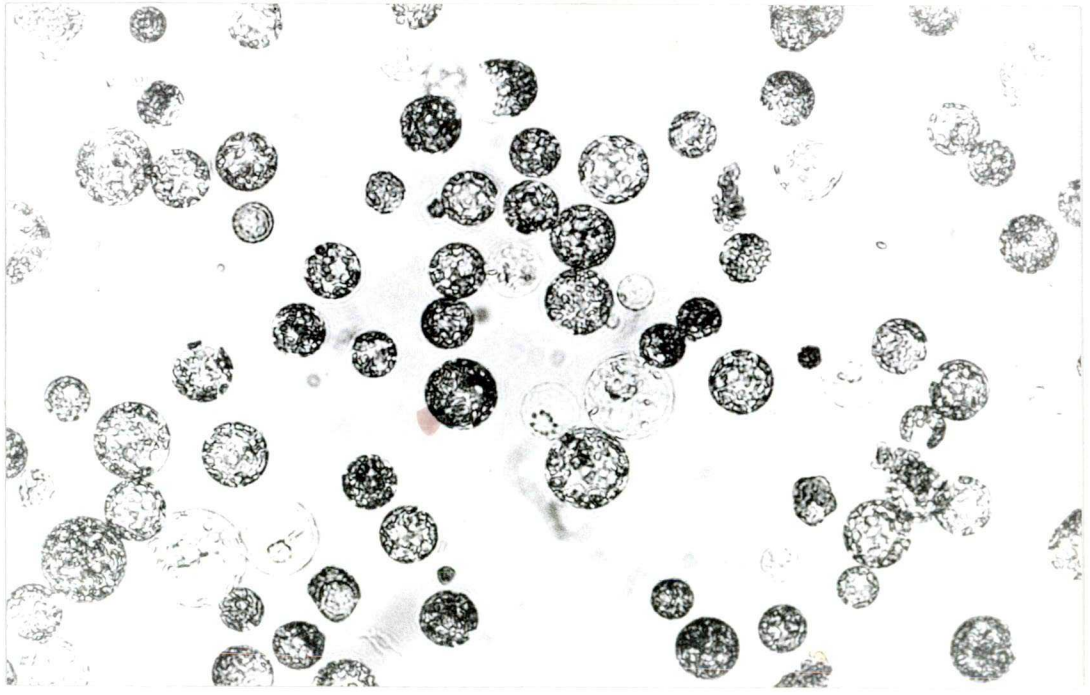

Figure 4. Freshly isolated leaf mesophyll protoplasts of *H.maximiliani*.

Table 2. Influence of hormone combinations on plating efficiency, colony formation and frequency of embryogenesis in *H. maximiliani* protoplasts culture.

Initial hormones[1]	Plating efficiency[2]	Hormones for colony growth and regeneration[3]	Colony formation efficiency[4]	Frequency of embryo-genesis[5]
1.0+1.0	22.0	1.0+1.0	3481 (8.7)	2368 (68)
		0.5 + 0.5	8602 (21.5)	5514 (64)
0.5 + 0.5	15.0	1.0 + 1.0	5112 (12.8)	4163 (81)
		0.5 + 0.5	4683 (11.7)	3393 (72)
0.1 + 0.1	7.5	1.0 + 1.0	48 (0.12)	0
		0.1 + 0.1	0	0

[1]NAA and BA concentrations (mg l^{-1}) in the initial liquid phase.

[2]Percent of protoplasts plated into the agarose droplets (4 x 10^4).

[3]NAA and BA concentrations (mg l^{-1}) in the liquid phase (colony growth) and in the MS (regeneration) medium.

[4]Number of colonies obtained, in parentheses percent of protoplasts initially plated.

[5]Number of embryogenic colonies, in parentheses percent of colonies obtained.

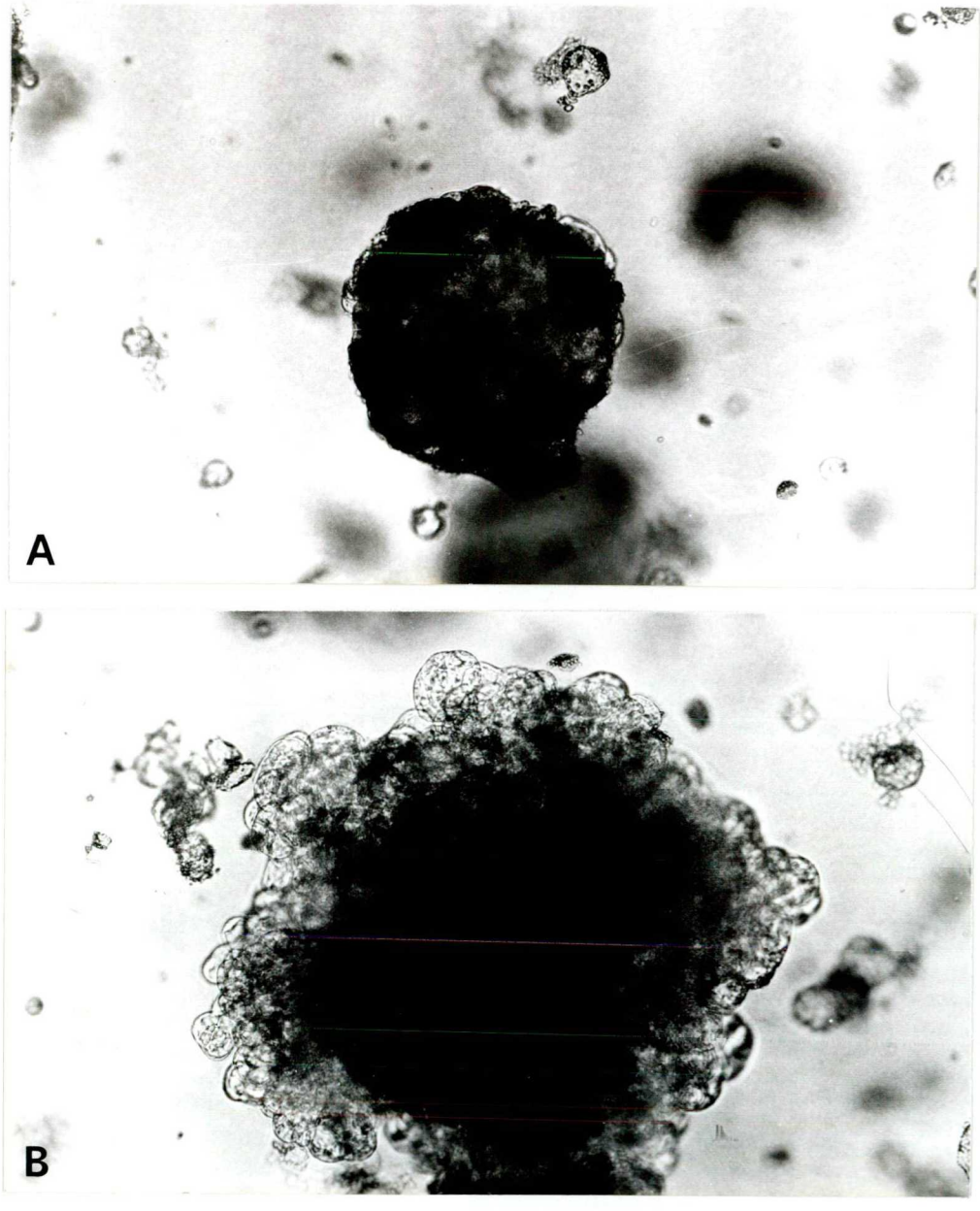

Figure 5. **A**, embryogenic and **B**, non-embryogenic protoplast derived colonies of *H.maximiliani*.

the same frequencies were obtained in three independent experiments.

The frequency of embryo formation was slightly higher (81% *vs* 68%, Table 2.) in cultures initiated using lower levels of hormones (0.5 + 0.5). However, this was offset by reduced plating efficiency and colony formation in previous stages of culture. The best results (in terms of the absolute numbers of embryogenic calli, Table 2.) were obtained when protoplast cultures were initiated at high concentrations of NAA and BA (1.0 + 1.0) and were subcultured using lower level of hormones (0.5 + 0.5). Although frequency of embryo formation was slightly higher in cultures initiated using lower levels of hormones (0.5 + 0.5), it was not sufficient to overcome the effect of reduced frequency of colony formation.

Callus tissues with mature embryos and embryogenic structures were transferred onto hormone free MS medium for germination (Fig.6A). Plantlets with well developed roots and shoots (about 2.5 to 3.0 cm long) were separated from the calli and were directly transplanted into sterilized soil. Normal development of these plants was observed in the greenhouse (Fig.6B). From every embryogenic callus we could obtain five or more normal plantlets. The frequency of plant regeneration, therefore, is practically the same as that of embryogenic calli.

In both studies on plant regeneration from protoplast cultures of *H.giganteus* and *H.maximiliani* described above, no data on fertility of regenerated plants have been presented. We have not aimed to reach the reproductive stage of regenerated plants because of following reasons: a) both wild species used in our study are perennial plants. It means seeds can be obtained only few years later after regenerated plants were transferred to soil; b) both these plant regeneration systems will not be utilized for direct plant regeneration from original species but for protoplast fusion products cultures in experiments on somatic hybridization between cultivated sunflower and one the wild species mentioned above.

Figure 6. **A**, plantlets of *H.maximiliani* developed on hormone-free medium and **B**, plant regenerated from protoplasts maintained in the greenhouse.

Summarized protocol of established plant regeneration system from leaf mesophyll protoplasts of *Helianthus maximiliani* Schrad.

a) embedding isolated leaf mesophyll protoplasts ($4x10^4$ prot/ml) in 1 ml of 0.25% agarose covered with 7-10 ml of liquid V-KM1 medium, culture them for 3 weeks with refreshing medium at 10 days interval, incubate in the dark at 25°C;

b) after 3 weeks replacing V-KM1 by V-KM2 medium and culture the protoplasts for another 3-4 weeks with refreshing medium at 10 days interval, incubate in the dark at 25°C;

c) release of colonies (1-2 mm in size) from agarose by gentle pipetting in 2-5 ml of V-KM and transfer them onto MS1 medium for 4 weeks, incubate in the light (1500 lux light intensity, 16 hours photoperiod) at 25°C;

d) transfer of embryos with part of callus tissue on MS2 medium for further embryo development;

e) transfer of shoots failed to develop roots on MS3 rooting medium;

f) transfer of plants with roots in soil, keeping them for the first 1-2 weeks under glass cover or plastic foil (to prevent fast evaporation) or maintain in vitro on maintenance medium.

For procedure of protoplast isolation and embedding protoplasts in agarose see Material and Methods, for media used in protocol see next page.

Media composition for protoplast culture and plant regeneration from leaf mesophyll protoplasts of *Helianthus maximiliani* Schrad.

	V-KM1	V-KM2	MS1	MS2
Macronutrients	V-KM	V-KM	MS	MS
Micronutrients	V-KM	V-KM	MS	MS
Vitamins (mg/l)				
Biotin	0.1	0.1	-	-
i-Inositol	100	100	100	100
Nicotinic acid	1	1	0.5	0.5
Ca-panthotenate	1	1	-	-
Pyridoxin-HCl	1	1	0.5	0.5
Thiamin-HCl	10	10	0.1	0.1
Glycine	-	-	2	2
Ascorbic acid	2	2	-	-
Cholin chloride	1	1	-	-
Folic acid	0.4	0.4	-	-
vit.A	0.01	0.01	-	-
vit.D_3	0.02	0.02	-	-
PABA	0.02	0.02	-	-
Organic constituents (g/l)				
Fructose	0.25	0.25	-	-
Mannitol	0.25	0.25	-	-
Ribose	0.25	0.25	-	-
Xylose	0.25	0.25	-	-
Mannose	0.25	0.25	-	-
Rhamnose	0.25	0.25	-	-
Cellobiose	0.25	0.25	-	-
Na-Pyruvate	0.02	0.02	-	-
Fumaric acid	0.04	0.04	-	-
Citric acid	0.04	0.04	-	-
Malic acid	0.04	0.04	-	-
Casein hydrolysate	0.25	0.25	-	-
L-Glutamine	1.0	1.0	-	-
Sorbitol	80	80	-	-
Sucrose	20	20	30	30
Coconut water (ml/l)	20	20	-	-
Hormones (mg/l)				
NAA	1.0	0.5	0.5	-
BA	1.0	0.5	0.5	-
Others				
MES (mg/l)	640	640	-	-
Agar (g/l)	-	-	6.0	6.0
pH	5.6	5.6	5.8	5.8

4.3. *Helianthus annuus*

Testing the regeneration ability of genotypes

Fourteen commercial sunflower hybrids as well as 77 inbred lines have been tested using the method of Paterson and Everett (1985). Regeneration ability was characterized by the average number of shoots developed on the hypocotyl segments cultured. Among more than 90 genotypes, only three hybrids and two inbred lines showed any response. The hybrid Florom-328 (from the Institute of Cereal and Industrial Plant Research, Fundulea, Romania) was the best responding genotype (0.6 shoots per hypocotyl explant), while Florom-206 and Blumix had average shoot numbers of 0.5 and 0.4, respectively. On the basis of this test Florom-328 was chosen for further studies.

Regeneration ability is apparently restricted to a small proportion of sunflower genotypes. Unfortunately, the genetic composition of the best genotype (Florom-328) is not known to us. However, it exhibits frequent branching of the stem when grown in the greenhouse. This trait which is not characteristic of cultivated sunflower may indicate contribution of genetic material from wild *Helianthus* species to this genotype. Wild species seem to be easier to regenerate *in vitro* (Chanabe et al. 1991; Bohorova et al. 1990; Krasnyanski et al. 1992; Polgar and Krasnyanski 1992). Thus, regeneration ability of the Florom genotype might have originated from a wild species used in the construction of this hybrid. The regenerable sunflower genotype of Burrus et al. (1991) PT024, designated in the paper as cms/*H.petiolaris*/cms HA89 backcross, may also contain input from a wild species.

Identification of factors affecting plating efficiency and shoot formation

In the following discussion the term "shoot formation" refers to the typical way of plant regeneration (elongation of the shoot apex of somatic embryos without root development, see below).

The average yield of protoplasts from one gram of hypocotyl tissue was 2 to 3 millions. The protoplast population was rather heterogeneous regarding both their size and in the amount of cytoplasm (Fig.7A). A more homogeneous population was obtained when

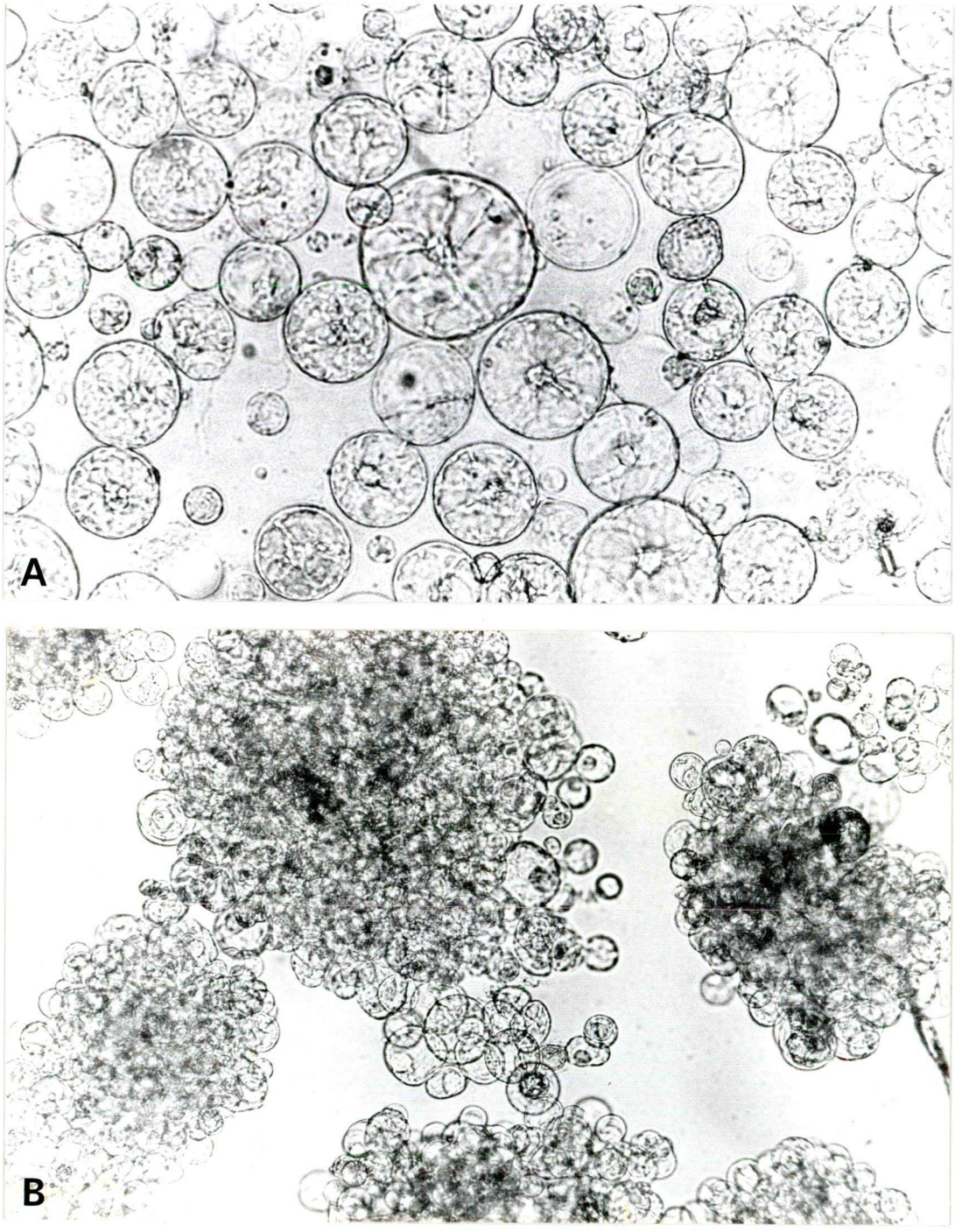

Figure 7. **A**, freshly isolated hypocotyl protoplasts and **B**, protoplast derived colonies in liquid culture of *H.annuus*.

large, highly vacuolated protoplasts were removed by filtration through nylon meshes with gradually decreased pore sizes. The age of hypocotyl material was a critical factor. Protoplast yield and plating efficiency were not significantly different when protoplasts from 5, 9 and 11 day-old material were used. However, somatic embryogenesis was observed only in colonies of protoplasts from 5 day old hypocotyl.

The medium for protoplast culture was V-KM (Binding and Nehls 1977) that proved to be satisfactory for sunflower protoplast culture. That has been proved in our preliminary experiments when media L_4 (Lenee and Chupeau 1986), MSb (Moyne 1988), K_3 (Nagy and Maliga 1976) supplemented with the same hormones have been used as well. Based on our previous studies, the agarose droplet culture of Shillito et al. (1983) was also used in the present investigation. Optimal protoplast density was determined first. The highest plating efficiency and colony formation was found at a density of 3 x 10^4 ml^{-1} (calculated using the volume of agarose droplet only). Fig.8A shows first divisions of protoplasts in droplets of V-KM medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA and solidified by 0.5 percent agarose. The protoplasts formed a cell wall within 2 days. Further divisions in most cases occurred inside the boundaries of the initial cell wall, thus the daughter cells became smaller than the original protoplasts and rich in cytoplasm. After one week the liquid over the droplets was replaced by fresh V-KM medium containing one-tenth of the original hormone concentrations. Further development of colonies followed different pathways. Developmental stages characteristic of somatic embryogenesis (Fig.8B to 8E) could be observed in the cultures. However, the frequency of shoot formation was rather low (0.18 percent of viable colonies formed).

In previous experiments on *Helianthus* protoplast culture, and also in preliminary experiments in this study, compact colonies representing the first stage of somatic embryogenesis could not be observed in liquid cultures (Fig.7B). It appears that a supporting matrix is essential for the development of embryos. For this reason, influence of the nature and concentration of gelling agent was investigated next. In cultures where 0.25 percent agarose was used, the frequency of shoot formation was only half of that observed in 0.5 percent agarose. Increasing agarose concentration to 1.0 percent resulted

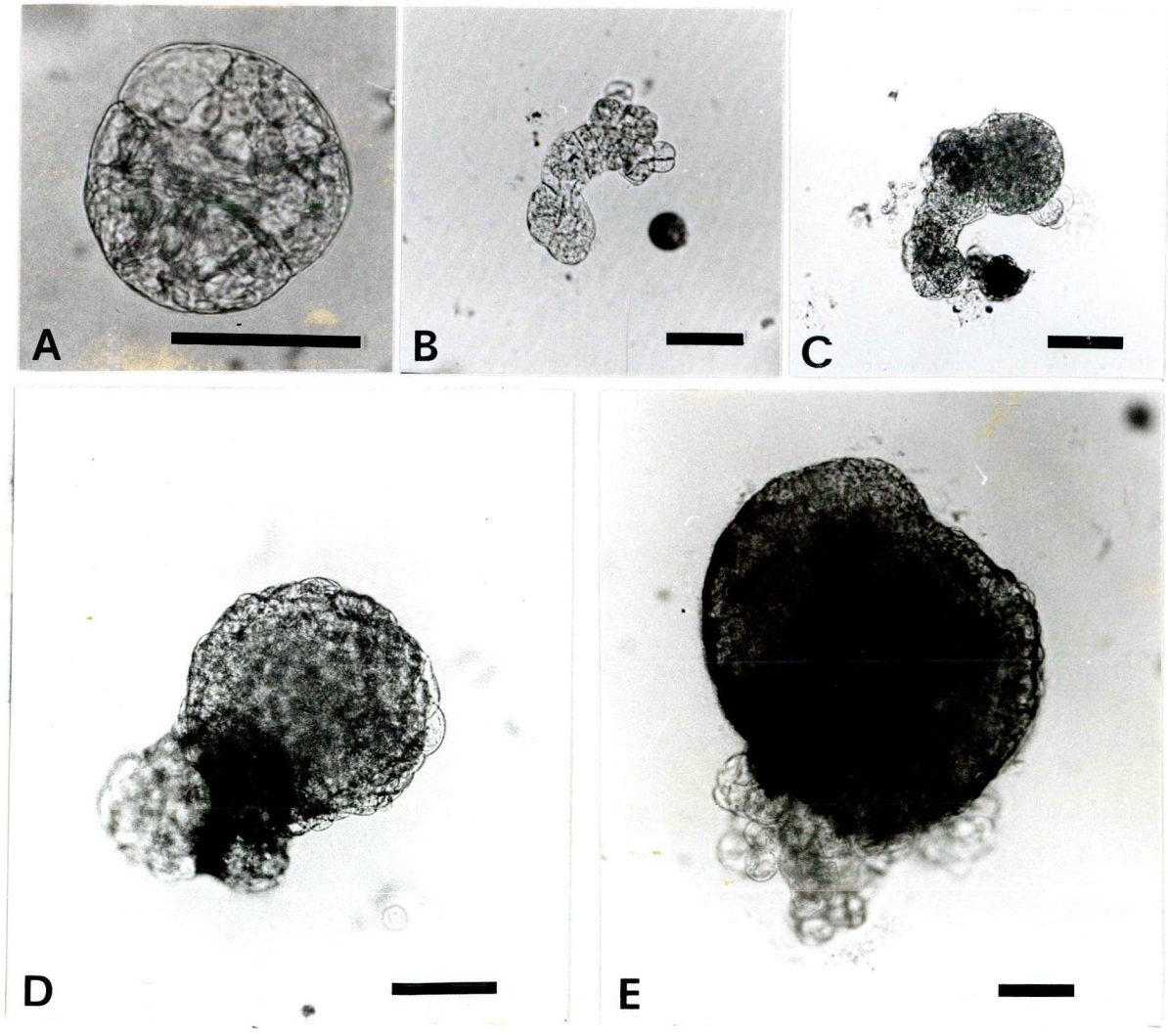

Figure 8. Development of protoplast-derived colonies in agarose droplets.
A, dividing protoplast after 4 days in culture, x 350, bar 50 μm; **B** and **C**, colonies after 10 and 12 days, x 75, bar 100 μm; **D**, colony after 15 days, x 80, bar 100 μm; **E**, globular embryo after 20 days, x 30, bar 200 μm.

in an increase of this frequency. However, due to the high rigidity of 1.0 percent gels, colonies could not be removed from the droplet without damage. Alginic acid at 1.4 percent concentration was also tried, but there was no improvement as compared to agarose solidified cultures.

In several dicotyledonous species including sunflower it has been shown that exposure of cultures to high concentrations of auxins for a limited period could induce subsequent development of somatic embryos (Finer 1987; Prado and Berville 1990). In our experiments NAA or 2,4-D treatment also improved embryogenic response of protoplast derived colonies. One week-old colonies grown in V-KM supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA were treated with different auxins by replacing the liquid medium over the droplets by V-KM supplemented with 2.2 mg l^{-1} of NAA, 2,4-D or dicamba. This medium was left on the droplets for one week, then it was replaced by V-KM with reduced NAA and BA contents as described above (V-KM 3). Treatment with dicamba, NAA and 2,4-D increased the frequency of shoot formation from 0.18 percent to 0.24, 0.8 and 1.8 percent, respectively (Fig.9).

The protoplast density (3 x 10^4 per ml) chosen in our starting experiments as optimal for initial division and colony formation appeared later to be too high for subsequent stages of colony development. Development of colonies slowed and they started to release a brown substance (probably polyphenols) into the medium. In order to provide sufficient environment for colony development, plating density was reduced to 5 x 10^3 prot. per ml inside the agarose droplet. The adverse effect of reduced density on plating efficiency was compensated by the presence of nursing protoplasts in the liquid phase of culture. These protoplasts were removed after one week when the liquid was refreshed. Plating efficiency and the frequency of colony formation were practically the same as those observed previously at higher protoplast densities. However, reduced density of colonies resulted in a significant increase in the frequency of shoot formation (1.2 *vs* 0.18 percent).

Influence of initial auxin concentration

Subsequent experiments were carried out using droplets solidified by 0.5 percent

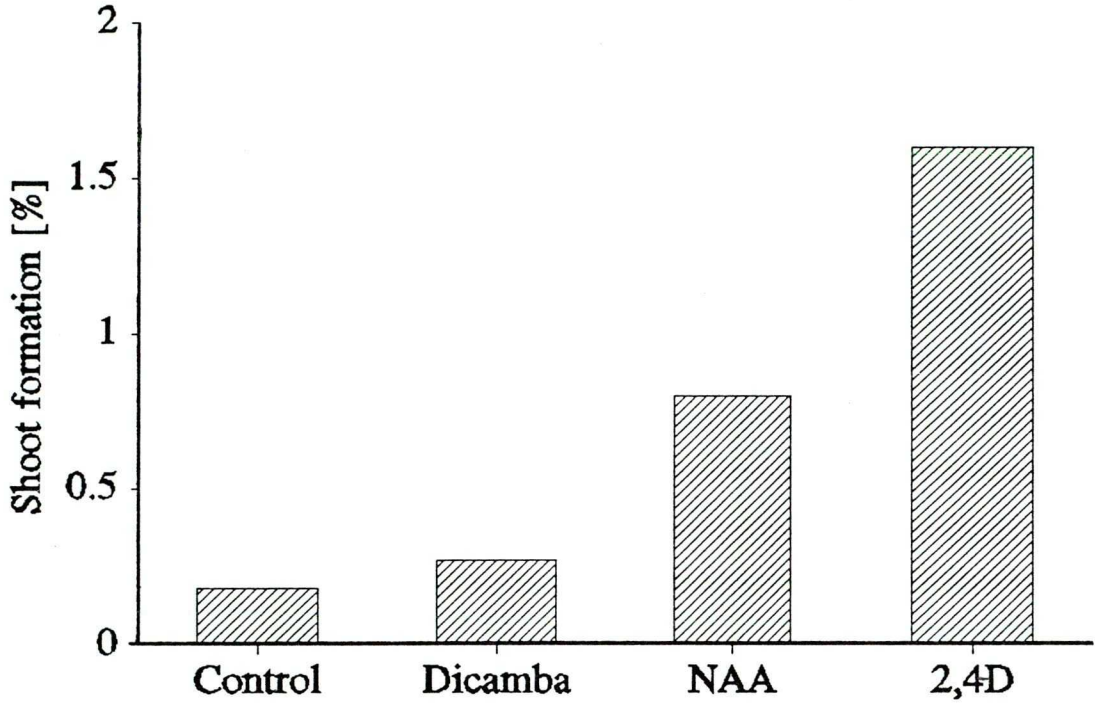

Figure 9. Effect of auxin treatment on the frequency of shoot formation in protoplast culture of *H.annuus*.

Dicamba, NAA and 2,4-D were presented in the liquid phase of cultures during the second week at a concentration of 2.2 mg l^{-1} . The **control** value indicates cultures where the liquid phase during the second week was the same as the initial one.

agarose, a protoplast density of 5 x 10^3 ml^{-1} and nursing protoplasts in the liquid phase at a density of 3 x 10^4 ml^{-1}. A systematic investigation on the influence of initial auxin concentration was performed. Protoplasts were cultured in the presence of different concentrations (0.5 to 10.0 mg l^{-1}) of NAA together with 2.0 mg l^{-1} BA. Interaction of

initial auxin concentration and subsequent 2,4-D treatment (2.2 mg l^{-1}) was studied by setting up parallel cultures with and without 2,4-D treatment for all NAA concentrations used. Results of these experiments are summarized in Fig.10. Plating efficiency (not shown) slightly decreased (from 34 to about 24 percent) with increasing NAA concentration. Colony formation was not significantly influenced by initial auxin levels. The frequency of shoot formation was highest at 4.0 mg l^{-1} initial NAA concentration with subsequent 2,4-D treatment. Under these conditions 9.6 percent of viable colonies formed shoots.

Embryo maturation and regeneration of plants

After three weeks of culture developing colonies were released from the agarose droplets and were cultured in liquid V-KM for a few days. They were then transferred onto solid MS regeneration medium and were exposed to light to induce maturation and germination of somatic embryos. Three media different in basic salt composition but otherwise supplemented with the same compounds (see Materials and methods) were tried at this point. On B_5 and White's media the colonies turned brown and developing shoots had very pale green color. On MS medium calli continued to grow vigorously and developing shoots had a dark green color. When agar was used as gelling agent, callus formation became predominant, while media solidified by Phytagel favored embryo development.

Within two to three weeks embryos at different developmental stages (from globular to torpedo-shaped) could be observed in the cultures. Development was slightly abnormal (Fig.11A, bottom row). Further development of both shoot and root was observed only in less than 5 percent of embryos. The typical way of development was shoot elongation accompanied by swelling or callusing at the root apex (Fig.11A, top row). Several attempts have been undertaken to initiate the normal development of both shoot and root apexes of embryos: 1) by changing BA concentration in protoplast culture procedure; 2) by optimizing MS regeneration medium composition. In first case only decrease of shoot formation efficiency and premature root development without shoot formation have been achieved. When regeneration medium was supplemented with the

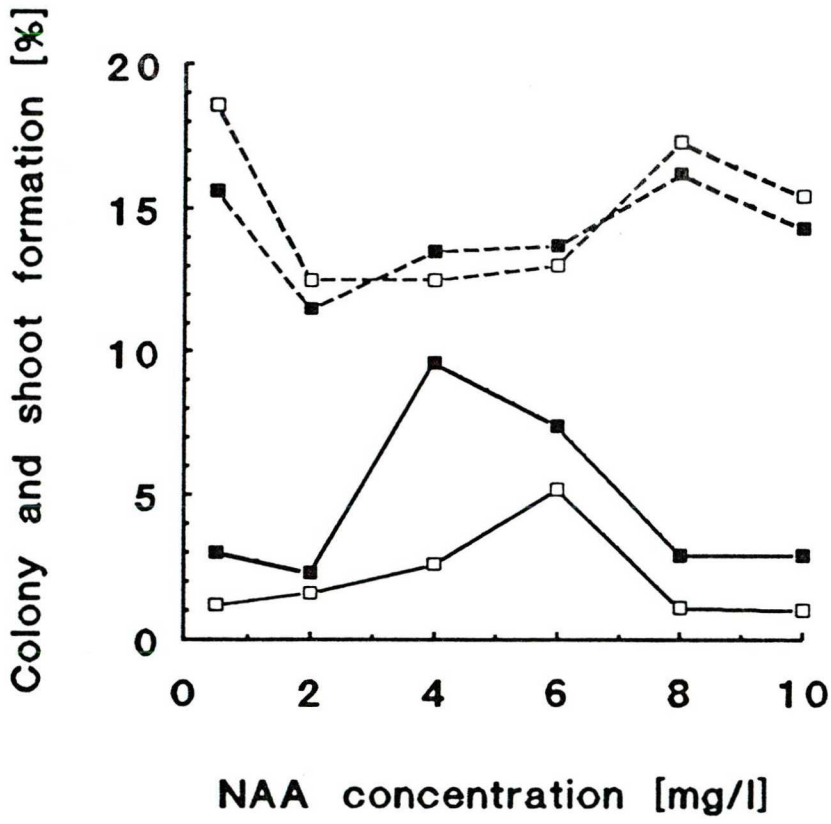

Figure 10. Effect of initial NAA concentration on colony and shoot formation.
Dashed lines, colony formation (percent of protoplasts plated);
solid lines, shoot formation (percent of viable colonies);
(■), with 2,4-D treatment;
(□), without 2,4-D treatment.

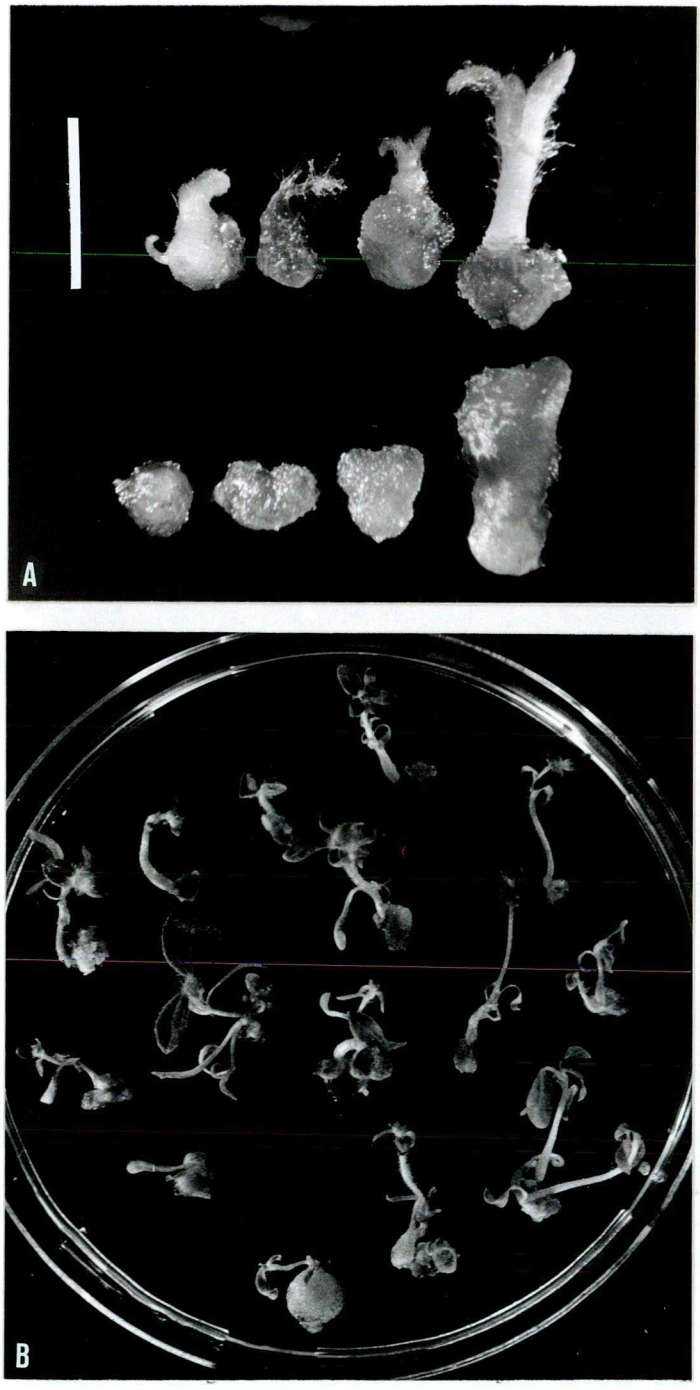

Figure 11. Development of embryogenic colonies on regeneration medium.

A, embryos at different stages of development, x 3, bar 5 mm; **B**, elongating shoots on hormone free medium in 9 cm petri dish.

same hormones, but osmoticum (sucrose) was added in higher concentrations (6 to 12 percent) with subsequent gradual decrease up to 3 percent the percentage of embryos with roots was increased (up to 8 percent) but the total shoot formation efficiency was dramatically lowered (0.6 percent of protoplasts plated).The decrease of BA concentration or its complete omission also did not have any improveming effect.

Development of normal shoots on the original MS regeneration medium (at a frequency of over 80 percent) occurred only when it was supplemented with silver nitrate, an inhibitor of ethylene action (Beyer 1976). In the absence of silver nitrate vitrification (elongated stem, pale green watery leaves) was observed and normal shoot formation was infrequent. Previously similar effects have been described and attributed to ethylene action in sunflower (Paterson et al. 1987; Chraibi et al. 1991; Chraibi et al. 1992) and also in other species (Chi and Pua 1989; Perl et al. 1988). It was essential to transfer the embryos onto hormone free MS medium as soon as shoot elongation had started. Shoots started to exhibit vitrification even in the presence of silver nitrate if they were left on the regeneration medium for a longer period. On hormone free medium the growth of calli stopped and normal shoot elongation continued (Fig.11B.).

Plantlets with well developed roots could be transplanted into soil. However, in the case of other shoots transplanting would have involved an additional period of rooting. Sunflower shoots in culture have a tendency of premature flowering (as noted e.g. by Burrus et al. 1991) which increases with the time elapsed in culture. The presence of flower buds in turn stops development of the shoots and causes senescence, usually without any seed production. Therefore, sunflower shoots should be transferred into soil at the earliest possible stage. An alternative to rooting is grafting of the shoot apex on *in vivo* grown hypocotyls (Fig.12A,B). Shoots as small as 10 mm in length could successfully be grafted. More than 80 percent of 111 shoots grafted were established on the rootstock and continued to develop. Although these plants did not grow to normal size, they flowered (Fig.12C) and produced seeds. The number of seeds obtained from individual plants was in the range of 5 to 10.

Figure 12. Grafting shoot tips on *in vivo* grown hypocotyls.

A, shoot developed on hormone free MS medium; **B**, same shoot grafted on hypocotyl; **C**, flowering regenerated plants.

Summarized protocol of established plant regeneration system from hypocotyl isolated protoplasts of *Helianthus annuus* L. (identified genotype cv.Florom-328).

a) embedding isolated hypocotyl protoplasts ($5x10^3$ prot/ml) in 1 ml of 0.5% agarose covered with 10 ml of liquid V-KM1 medium containing hypocotyl protoplasts of any genotype as "nurse" culture at density 3 x 10^4 prot/ml, culture for 1 weeks, incubate in the dark at 25°C;

b) replacing V-KM1 medium with "nurse" culture by V-KM2 and culture protoplasts for another 1 week, incubate in the dark at 25°C;

c) replacing V-KM2 by V-KM3 medium and culture protoplasts for 1 week, incubate in the dark at 25°C;

c) release of colonies from agarose by gentle pipetting in fresh V-KM3 medium and leave colonies in a liquid for 3-5 days, incubate in the dark at 25°C;

d) plating colonies on MS1 regeneration medium for embryo development;

e) transfer of developed embryos with small shoots (2-3 mm length) on MS2 medium for shoot elongation;

f) cut off unrooted shoots from callus tissues and grafting them in vivo on hypocotyls germinated in sterilized soil;

g) shoots grafted on hypocotyls and normal plants with roots transferred in soil for first 1-2 weeks should be kept under the glass cover or plastic foil (to prevent fast evaporation);

For procedure of protoplast isolation and embedding protoplasts in agarose see Material and Methods, for media used in protocol see next page.

Media composition for protoplast culture and plant regeneration from hypocotyl isolated protoplasts of *Helianthus annuus* L.

	V-KM1	V-KM2	V-KM3	MS1	MS2
Macronutrients	V-KM	V-KM	V-KM	MS	MS
Micronutrients	V-KM	V-KM	V-KM	MS	MS
Vitamins (mg/l)					
Biotin	0.1	0.1	0.1	-	-
i-Inositol	100	100	100	100	100
Nicotinic acid	1	1	1	0.5	
Ca-panthotenate	1	1	1	-	-
Pyridoxin-HCl	1	1	1	0.5	-
Thiamin-HCl	10	10	10	0.1	0.2
Ascorbic acid	2	2	2	-	-
Cholin chloride	1	1	1	-	-
Folic acid	0.4	0.4	0.4	-	-
vit.A	0.01	0.01	0.01	-	-
vit.D_3	0.02	0.02	0.02	-	-
PABA	0.02	0.02	0.02	-	-
Organic constituents (g/l)					
Fructose	0.25	0.25	0.25	-	-
Mannitol	0.25	0.25	0.25	-	-
Ribose	0.25	0.25	0.25	-	-
Xylose	0.25	0.25	0.25	-	-
Mannose	0.25	0.25	0.25	-	-
Rhamnose	0.25	0.25	0.25	-	-
Cellobiose	0.25	0.25	0.25	-	-
Na-Pyruvate	0.02	0.02	0.02	-	-
Fumaric acid	0.04	0.04	0.04	-	-
Citric acid	0.04	0.04	0.04	-	-
Malic acid	0.04	0.04	0.04	-	-
Casein hydrolysate	0.25	0.25	0.25	0.5	-
L-Glutamine	1.0	1.0	1.0	-	-
Sorbitol	80	80	80	-	-
Sucrose	20	20	20	30	30
Coconut water (ml/l)	20	20	20	-	-
Hormones (mg/l)					
NAA	4.0	-	0.05	0.01	-
BA	2.0	-	0.2	0.2	-
2,4-D	-	2.2	-	-	-
GA_3	-	-	-	0.1	-
Others					
$AgNO_3$	-	-	-	1.0	-
MES (mg/l)	640	640	640	-	-
Phytagel (g/l)	-	-	-	3.0	2.0
pH	5.6	5.6	5.6	5.8	5.8

5. CONCLUSIONS

Based on the above results it can be concluded that:

1. The age of donor plants is a critical factor for the quality of both mesophyll and hypocotyl protoplasts. In the case of cultivated sunflower it also influences the frequency of embryogenesis.

2. Embedding protoplats in a rigid matrix (agarose) covered by a liquid phase is essential to obtain colonies capable of plant regeneration. For the initial culture of *Helianthus* protoplasts V-KM medium appears superior to others.

3. The use of nursing protoplasts in the liquid phase allows significant reduction of initial plating density. Reduced density in turn increases the frequency of colonies capable of embryogenic development.

4. MS medium is superior to B_5 or White for maturation and germination of somatic embryos. The use of an ethylene inhibitor, silver nitrate, in the regeneration and maturation media is essential for decreasing vitrification of regenerated shoots.

5. The problem of premature flowering of regenerated sunflower shoots can be overcome by the use of grafting to hypocotyls of *in vivo* grown plants.

6. Development of regeneration systems in *Helianthus maximiliani* and *Helianthus giganteus* provides the possibility of selecting somatic hybrids of these species and cultivated sunflower after protoplast fusion.

7. Development of a practically useful regeneration system from protoplasts of cultivated sunflower makes production of genetically transformed plants feasible (*via* direct DNA uptake into protoplasts and subsequent plant regeneration).

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