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PLANT BIOTECHNOLOGY AND SUNFLOWER IMPROVEMENT

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SUMMARY

Plant biotechnology has given new hopes for crop improvement with the major advances in tissue culture and recombinant DNA technology. Sunflower appears to be very responsive to micro-propagation, which could be commercially exploited for the multiplication of rare hybrids and male sterile lines. Culturing of immature embryos helps in transferring desirable genes from wild species where post-fertilization incompatibility otherwise leads to embryo/endosperm degeneration. Regeneration of whole plants from somatic cell cultures of sunflower seems rewarding for the induction and selection of mutants at cellular level. High frequency shoot regeneration from cultured immature cotyledons could be hopefully exploited in Agrobacterium tumefaciens mediated transformation studies. In vitro production of haploids, using anther/pollen culture helps in developing homozygous true breeding lines in a very short period but has not been accomplished in sunflower. Transfer of phaseolin gene from bean to sunflower via recombinant DNA technology, demonstrates its suitability for transferring cloned genes imparting resistance to diseases, insect pests and herbicides.

INTRODUCTION

Sunflower, Helianthus annuus L. is a member of the compositae and is cultivated world-wide commercially for edible oil. The oil is rich in unsaturated fatty acids, contains large amounts of vitamin E and is easy to refine. The major breeding objectives include; increase in seed yield, oil content, insect and disease resistance and decrease in plant height. The sunflower breeding has been somewhat difficult as it is essentially a cross-pollinated crop. Besides, it shows a wide range of self-incompatibility, which creat difficulty in obtaining true breeding homozygous lines. Using conventional methods like introduction, selection, population and heterosis breeding, a series of high yielding, widely adapted disease resistant with improved quality and increased tolerance to soil and environmental stresses have been developed. Plant biotechnology offers the most potent emerging techniques for the induction as well as utilization of genetic variability for desirable agronomic traits. In the recent years rapid advances in tissue culture, pollen and protoplast culture, genetic engineering using vector and vectorless systems have made possible, the genetic manipulation at cellular levels. Some of the molecular biology techniques like in-situ DNA hybridization and Restriction fragment length polymorphism (RFLP's) are increasingly being used for the precise characterization of the germplasm at molecular levels.

In the present paper the various aspects of sunflower cell, tissue, organ, protoplast culture and genetic engineering, are reviewed (Table 1) and the importance of these methods in the improvement is emphasized.

SHOOT TIP CULTURE

Shoot-tip culture, ensures true to type, rapid and large scale multiplication of rare and important germplasm.Moreover, culturing of shoot meristems (0.2-0.5 mm) helps in disease free multiplication. Many pathogens are eliminated and the resulting plants show uniformity, improved vigour and quality. In 1954, Hendrikson cultured H. annuus shoot tips with or without cotyledons on modified White's medium in order to study flowering in a variety 'Mammoth Russian'. Whole plants were obtained and flowering occurred in culture within three months. Paal et al. (1981) examined the effect of plant hormones on in vitro plantlet regeneration from cultured shoot tips. Shoot tips of a variety 816/b were cultured on MS with various combinations of NAA and BA. BA alone or with NAA tended to promote callusing, while NAA alone allowed the shoot tip to grow into a plant. Resulting plants were successfully transferred to green house and plants thus grown showed early flowering. Likewise Trifi et al. (1984) developed a method for shoot multiplication of two varieties Issanka and Record from a single hybrid Blueberry. The method required the growing of shoot tips on low concentrations of NAA and BA (0.5 mg/l each), which allowed a shoot to grow out. The nodes were then separated and recultured, flowering was observed in cultures within 6 weeks. Paterson (1984) conducted extensive studies on flowering and development of adventitious/multiple shoots from cultured shoot tips of 111 inbreds of H. annuus. Shoot multiplication was optimal from half shoot apices cultured on MS with 0.1-1.0 mg/l of BA or kinetin. Whereas auxins, inhibited the multiplication and promoted callusing. Rooting was poor and was not promoted by auxins. Flowering of multiple shoots was observed after 3 weeks of culture. A number of plant growth regulators and environmental conditions had no effect on flowering which supported the determinate apex theory of sunflower. Adventitious shoots were induced on leaves of the multiple shoots in some inbreds.Therefore, the shoot tip culture method can be successfully used for international exchange of disease free germplasm; to multiply inbreds, male sterile lines and genetically engineered plants, however, efforts are required to check early flowering, to ensure rapid shoot multiplication over a longer period.

EMBRYO CULTURE

Wild species of the cultivated crop plants are useful reservoirs of genetic variability for various economic traits like quality, disease and pest resistance, tolerance to stress environments and male sterility etc. To incorporate such traits into cultivated varieties, involve hybridization between diverse parents. However, in such crosses several crossability barriers are encountered. Incompatibility between the embryo and endosperm is the major problem which results in the abortion of hybrid embryos. Under such situations, embryo culture can be successfully used in obtaining hybrids among otherwise hard-to-cross species. In sunflower, Chandler and Beard (1983) successfully hybridized 53 combinations of species including 21 which were not achieved through conventional methods. Hybrid embryos, 3-7 days after pollination were excised and cultured on B5 medium. Embryos initially developed on a solid medium containing inorganic components, vitamins, amino acids and sucrose (120 g/l). For embryo germination and seedling growth the cultured embryos were transferred to a liquid medium

containing only inorganics and sucrose (10 g/l). Likewise new hybrids were obtained from H. annuus x H. hirsutus and H. scaberimus x H. annuus combinations by culturing hybrid embryos on modified White's medium (Bohorova et al., 1985). Shoots from resulting hybrids were successfully propagated on shoot inducing medium supplemented with glutamine (800 mg/l) and asparagine (800 mg/l). In sunflower some of the diploid perennial species carry resistance to diseases and pests, which are difficult or impossible to cross with the cultivated sunflower using conventional methods. Therefore, the techniques of embryo culture could be hopefully exploited to transfer desirable genes accross the species barrier.

ANTHER CULTURE

The production of haploids by the in vitro culture of excised anthers and isolated pollen has created considerable interest among geneticists and plant breeders. The absence of dominance effects in haploids, facilitate the genetic studies regarding the inheritance of various traits. Furthermore, in view of their great importance in detecting mutations, obtaining homozygous diploids, evolutionary studies and analytical method of breeding, they are required in large numbers. Using anther and/or pollen culture, haploids have been obtained in a wide variety of platns (Bajaj, 1983). In sunflower, very limited work has been done on the in vitro culturing of excised anthers/pollen. Alissa et al. (1985) have reported androgenesis from the cultured anthers of Helianthus. Similarly, Bohorova et al. (1985) have reported best callus formation from the anthers of many species on MS with 2,4-D (1 mg/l) and kinetin (0.2 mg/l). However, calli thus obtained lacked subsequent plant regeneration. Direct shoot regeneration from the anthers of H. divaricatus and H. annuus x H. deeapetalus was observed on MS containing zeatin (5 mg/l). One-third of the anther derived plants showed 2n = 34, whereas 2/3rd had 45-51, 68, 102 chromosomes. After eight subcultures, all regenerants had a chromosome count of 2n=51, identical to the donor hybrid plant. In sunflower, concerted efforts are required to obtain haploids in large numbers for developing homozygous true breeding lines for population and heterosis breeding programmes.

ESTABLISHMENT OF TISSUE CULTURE AND PLANT REGENERATION

Efficient plant regeneration particularly from long term maintained callus/cell suspension cultures, is required for the induction of somaclonal variation and for successful gene transfer using various vector and vectorless systems. In sunflower, Sadhu (1974) made some preliminary studies to know its hormal requirements for dedifferentiation as well redifferentiation. Stem pith tissue, cultured on modified White's medium containing IAA (1 mg/l) exhibited callusing and subsequent differentiation. Resulting plant survived when transferred to the soil. Shoot organogenesis from the callus resulting into fertile shoots, have been observed for two sterile hybrids (Georgieva-Todorova et al., 1980). Best response was observed on MS containing BA (0.1 mg/l), NAA (0.1 mg/l), GA_3 (0.01 mg/l) and adenine sulfate (40 mg/l). Greco et al. (1984) studied the effect of 2,4-D and 6-BAP on callus induction and plant regeneration. Leaf and cotyledon pieces, shoot apices and hypocotyl segments of sunflower were grown on MS supplemented with several concentrations of 2,4-D and/or 6-BAP. Response differed according to the nature

of the explant and the hormonal treatment. 6-BAP induced better callus from the different explants. 2,4-D alone induced poorly developed, nodular callus, whereas 6-BAP alone induced abundant growth of compact, green callus.Several of the calli induced by 6-BAP regenerated many shoots, some of which flowered in vitro. Likewise, shoot apices and stem pith tissue of hybrid among H. annuus x H. decapetalus, cultured on MS containing NAA (0.1 mg/l), BAP (0.1 mg/l), GA (0.01 mg/l) and adenine sulfate (40 mg/l) exhibited callusing and shoot regeneration (Bohorova et al., 1985). Moreover, somatic embryogenesis and plant regeneration have been achieved in some inbreds from the hypocotyls cultured on MS containing 6.9 g/l total KNO_3, adenine sulfate (40 mg/l), casaminoacids (500 mg/l), BA (1.0 mg/l), NAA (1.0 mg/l) and 0.1 mg/l GA_3 (Paterson and Everett, 1985). Hypocotyls and cotyledons from hybrid Cargill S500 were cultured on MS and Miller's media with kinetin (0.5 mg/l) and 2 mg/l of either NAA, IAA, 2,4-D and picloram (Piubello and Caso, 1986). Some of the media tested for differentiation from the cotyledon calli yielded meristamatic nodules, roots and bud like structures. Globular embryoids were formed on medium with kinetin (5.0 mg/l). Ethylene is an important factor in the differentiation of cultured plant cells. In this regard hypocotyl-derived callus from H. annuus L. inbred line SS415B regenerated significantly more plants if the seedlings were grown in light (Robinson and Adams, 1987). Treating 3-day-old, dark-grown seedlings with 10 µM aminoethoxyvinylglycine (AVG) effectively inhibited ethylene production for at least 7 days. Hypocotyl callus derived from AVG-treated seedlings gave the same amount of regeneration as callus from light grown seedlings. Thus callus from either light grown seedlings or dark grown seedlings treated with AVG, showed significantly more regeneration than callus derived from dark grown seedlings.

Direct shoot development in H. annuus inbreds and hybrids from cultured cotyledons of zygotic embryos has also been achieved (Power, 1987). Dormant mature and immature zygotic embryos were dissected and cultured on modified MS medium supplemented with BA (0.1 mg/l), NAA (0.5 mg/l) adenine sulfate (40 mg/l) and casaminoacids (500 mg/l). For certain inbreds and hybrids adventitious shoot formation occurred from callused cotyledonary tissue, particularly along the cut edges. The developmental stage of the zygotic embryo was critical. Eighty per cent of immature H. annuus, Mammoth Russian embryos produced adventitious shoots from cotyledons, while mature embryos did not. Rooted shoots were successfully grown to maturity in a greenhouse. Therefore, the reliable plant regeneration techniques directly from the explants or callus/cell suspension cultures could be hopefully exploited for obtaining transgenic sunflower plants.

We studied the callus induction and its subsequent proliferation in two hybrids, viz. NS-H-26-RM and BSH-1 from root, hypocotyl and cotyledon segments cultured on Murashige and Skoog (1962) medium containing different combinations and concentrations of 2,4-D, NAA and BAP. Callus formation was observed on all the six media (Table 2), however, better proliferation was observed only on S4 and S6. Callus formation, generally from cut ends, was observed after 5 days of culturing. Addition of only 2,4-D or BAP lead to the development of very slow growing calli. The size and the nature of the explant was found to affect the callus formation (Table 3). Explants less than 3 mm usually senesced, whereas those ranging between 5-7 mm were potentially better. Both hypocotyl and cotyledon explants

Table 1. Protoplast, Cell, Tissue and Organ Culture studies on sunflower

Genotype	Explant	Medium	Growth Response	Reference
Shoot Tip Culture				
Helianthus annuus var. Mammoth Russian	Shoot tips with or without cotyledons	Mod. White's	Whole plants, flowered in culture within 3 months	Hendrickson (1954)
H. annuus var.816/b	Shoot tips	MS with NAA and BA	Full grown plants, early flowering	Paal et al. (1981)
H. annuus vars. Issanka and Record	Shoot tips	Medium with NAA and BA (0.5 mg/l each)	Full grown plants, flowered in culture within 6 weeks	Trifi et al.(1981)
H. annuus 111 inbreds including 89B and SS 405B	Shoot tips from 3-4 day old seedlings	MS with BA or Kinetin (0.1-1.0 mg/l)	Multiple shoots, flowered after 3 weeks of culturing. Adventitious shoots were induced on leaves of some inbreds	Paterson (1984)
Embryo Culture				
H. annuus and wild species	Hybrid embryos	B5 medium 1. Embryo growth medium with inorganics, vitamins, amino acids and sucrose (120 g/l). 2. Germination medium - liquid medium containing only inorganics and sucrose (10 g/l).	Full grown hybrid plants	Chandler and Beard (1983)
H. annuus x H.hirsutus H. scaberimus x H. annuus	Hybrid embryos	Mod.White's medium	Full grown plants	Bohorova et al. (1985)
Anther Culture				
Wild species and interspecific hybrids of Helianthus	Anthers	-	Androgenesis	Alissa et al.(1985)
H.divaricatus H.annuus x H. deeapetalus	Anthers	MS with 2,4-D(1 mg/l) and Kinetin (0.2 mg/l)	Androgenesis	Bohorova et al. (1985)

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Establishment of Tissue Culture and Plant Regeneration

A. Callus Induction and Plant Regeneration

Genotype	Explant	Medium	Growth response	Reference
H. annuus	Stem pith	Mod.White's with IAA (1 mg/l)	Friable callus differentiated into whole plant	Sadhu (1974)
H. annuus sterile hybrids	Stem segments	MS with BA (0.1 mg/l), NAA (0.1 mg/l),GA_3 (0.01 mg/l) and adenine sulfate (40 mg/l)	Shoot regeneration	Georgieva-Todorova et al. (1980)
H. annuus var. Sannace (synthetic)	Hypocotyls cotyledons,leaves and shoot apices	MS with BAP and/or 2,4-D (induction medium) MS with BAP (1 mg/l) (regeneration medium)	Shoot formation directly from explants and from callus	Greco et al. (1984)
H. annuus x H. decapetalus (hybrid)	Stem pith and shoot apices	MS with NAA (0.1 mg/l), BAP (0.1 mg/l), GA (0.01 mg/l) and adenine sulfate (40 mg/l)	Shoot regeneration	Bohorova et al.(1985)
H. annuus (inbreds)	Hypocotyls from 12 day old seedlings	MS with 6.9 g/l total KNO_3, adenine sulfate (40 mg/l), casamino acids (500 mg/l), BA (1 mg/l), NAA (1.0 mg/l) and GA_3 (0.1 mg/l)	Embryogenesis and shoot regeneration	Peterson and Everett (1985)
H. annuus Cargill S 500 (hybrid)	Hypocotyls, cotyledons	MS and Miller's (1963) with kinetin (0.5 mg/l) and 2 mg/l of either NAA, IAA, 2,4-D and picloram.	Callus and embryoids	Piubello and Caso (1986)
H. annuus hybrids NS-H-26-RM, BSH-1	Roots,hypocotyls cotyledons	MS+NAA (2 mg/l) + BAP (0.5-1 mg/l)	Smooth and nodular calli	Vasiljevic et al. (unpublished)

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Genotype	Explant	Medium	Growth response	Reference
H. annuus inbred SS 415B	Hypocotyls from light grown seedlings or from AVG treated dark grown seedlings	HaR medium (MS salts and vitamins, 49.5 mM additional KNO_3, 5.4 µM NAA, 4.4 µM BA, 0.3 µM GA, 0.05% w/v casamino acids, 217 uM adenine sulfate, 88 mM sucrose.	Treatment of dark grown seedlings with AVG promoted shoot regeneration in the resulting calli	Robinson and Adams (1987)
		B. Direct Shoot Regeneration		
H. annuus hybrids SIGCO H0849, 455, 468 and 461D Mammoth Russian	Cotyledons from immature and mature embryos	MS with BA (0.1 mg/l), NAA (0.5 mg/l) adenine sulfate (40 mg/l) and casamino acids (500 mg/l)	Adventitious shoots from immature cotyledons	Power (1987)
		Protoplast Culture		
Genotype	Source of protoplast	Culture method	Growth response	Reference
H. annuus	Hypocotyls	Agarose bead method	Protoplast divided and formed micro-calli	Bohorova et al. (1986)
H. praecox	Leaves			
H. annuus	Hypocotyls	Plating (Shillito et al., 1983) method	Direct embryogenesis	Dupuis and Chagvardieff (1987)

Table 2. Percentage callus induction from hypocotyls of sunflower cultured on six media

Medium	Genotype	NS-H-26-RM			BSH-I		
		No.of explants cultured	No. of callusing explants	Percentage callusing	No.of explants cultured	No. of callusing explants	Percentage callusing
S_1 = MS+2,4-D (2 mg/l)		14	14	100.0	16	16	100.0
S_2 = MS+BAP (3 mg/l)		16	14	87.7	9	8	88.8
S_3 = MS+BAP (5 mg/l)		15	15	100.0	13	13	100.0
S_4 = MS+NAA (2 mg/l) + BAP (0.5 mg/l)		17	17	100.0	16	15	93.7
S_5 = MS + BAP (10 mg/l)		15	13	86.6	16	15	93.7
S_6 = MS+NAA (2 mg/l) + BAP (1 mg/l)		16	16	100.0	16	16	100.0

Table 3. Percentage callus induction from root, hypocotyl and cotyledon segments of sunflower cultured on MS + NAA (2 mg/l) + BAP (0.5 mg/l)

Genotype	NS-H-26-RM			BSH-I		
	No. of explants cultured	No.of callusing explants	Percentage callusing	No.of explants cultured	No.of callusing explants	Percentage callusing
Root	15	13	86.6	16	13	81.2
Hypocotyl	17	17	100.0	16	15	97.3
Cotyledon	18	18	100.0	20	20	100.0

produced better callus whereas root-derived callus in many instances showed browning and poor proliferation. Hypocotyl derived callus appeared very soft, smooth and light green in colour. Whereas, cotyledon explants produced somewhat hard, nodular and green callus. Upon subculturing onto basal or medium containing decreased levels of NAA and BAP, calli showed rooting and the formation of dark green meristamatic nodules resembling shoot buds.

PROTOPLAST CULTURE

The technology of protoplast is now being regarded as a perspective tool for the induction of genetic variability in crop plants. The upsurge of interest in this field is mainly because of the potential possibilities for parasexual manipulations, i.e. (i) production of amphidiploids, (ii) transfer of partial nuclear information, (iii) active participation of male cytoplasm (production of cybrids), and (iv) gene transfer using microinjection and electroporation. In sunflower protoplasts have been isolated from seedling hypocotyls and leaves of axenic shoot cultures (Bohorova et al., 1986; Bohorova, 1987). The cultured protoplasts have been shown to form micro calli (Lenee and Chupeau, 1986; Bohorova et al., 1986) and direct somatic embryos (Dupuis and Chagvardieff, 1987). Maximum plating efficiency was at a density of 5×10^4 protoplasts/ml in Difco bacto agar with NAA (0.5 mg/l) and using hybrid 76-D. Reliable plant regeneration from sunflower protoplasts is, however, still to be achieved.

GENETIC ENGINEERING

Being a dicot, sunflower is susceptible to Agrobacterium mediated gene transfer. A number of genes have been transferred, for instance phaseolin gene from bean (Murai et al., 1983), maize gene into sunflower (Goldsbrough et al., 1986), Moreover, full grown transgenic sunflower plants possessing kanamycin resistance have also been obtained (Everett et al., 1987). These results demonstrate that introduction of foreign genes via Agrobacterium mediated transformation can be hopefully used for the agronomic improvement of sunflower.

CONCLUSIONS

Rapid developments in the area of tissue culture and genetic engineering hold significant promise as a valuable adjunct to the sunflower improvement. There is a considerable hope for cloning inbreds and male sterile lines through shoot tip culturing and/or via somatic embryogenesis. Wide hybridization through embryo culture and hybrid callus would enable the incorporation of desirable characters and would presumably enhance the rate of introgression of useful alien genes. Concerted efforts are required to develop suitable procedures for obtaining haploids through anther/pollen culture. Plant regeneration ability from callus cultures could be hopefully exploited for the induction of somaclonal variation and gene transfer using plasmids, microinjection and electroporation methods. Somatic hybridization can be used to transfer male sterility into desirable breeding lines, however, more efforts are required to achieve reproducible plant regeneration from cultured protoplasts. It is envisaged that during the next few years, biotechnology will play very important role for the generation of genetic variability and sunflower improvement.

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