Institut für Molekulare Physiologie und Biotechnologie der Pflanzen

Genetic transformation of two high oleic *Helianthus annuus* L. genotypes using different transformation methods

Inaugural – Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Hohen Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

Bonn

vorgelegt am 26.01.2005

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This work was carried out at the Institute of Plant Molecular Physiology and Biotechnology, University of Bonn, Germany under the supervision of Prof. Dr. H. Schnabl.

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Tag der mündlichen Prüfung: 22.03.2005

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

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IN ADVANCE PUBLICATIONS OF THE DISSERTATION

Parts of the results of this study have been published in advance by permission of the Faculty of Agriculture, represented by the supervisor of this study.

Research Paper

Sh. Mohamed, P.C. Binsfeld, C. Cerboncini and H. Schnabl, 2003. Regeneration systems at high frequency from high oleic *Helianthus annuus* L. genotypes. J. App. Bot., 77: 85-89.

Sh. Mohamed, R. Boehm, P.C. Binsfeld and H. Schnabl, 2004. *Agrobacterium*-mediated transformation of two high oleic sunflower *(Helianthus annuus* L.) genotypes: assessment and optimization of important parameters. Helia, Novi Sad, vol. 27, No. 40: 25-40.

Research Presentations

Mohamed, Sh., Boehm, R., Binsfeld, P.C. and Schnabl, H. Assessment and optimization of parameters enhancing *Agrobacterium*-mediated transformation of two high oleic *Helianthus annuus* L. genotypes. Poster presented at (SUNBIO 2003) Sixt European Conference on Sunflower Biotechnology, Seville, Spain, 5-9 October 2003.

ACKNOWLEDGEMENT

It is a pleasure to express my sincere and deepest heartfelt gratitude to my **Prof. Dr. H. Schnabl** for her kind supervision, continuous encouragement, valuable enthusiastic discussion and unfailing advice throughout the present work.

I wish to express my sincere thanks for **Dr. R. Boehm** for his invaluable supervision and critical reading of the manuscript.

I am also grateful to **Prof. Dr. M. Janssens** for his kind acceptance as co-referent for my thesis.

I also thank all the members of the Institute of Plant Molecular Physiology and Biotechnology, University of Bonn, especially **Dr. C. Cerboncini** for his enthusiasm dealing with computer problems and his help in Germany translation.

I would like to express my special thanks to **Frau Kriegs, Frau Beine and Frau Becker** for their kindness help to translate part of my thesis from English into German.

I am extremely grateful for my husband **Prof. Dr. Ahmed Abd El-Gaffar Darwish** for his encouragement, support, help and comfortable atmosphere during the long hard work of this thesis. To my pretty daughters **Nada, Nadin** and **Nermin**, for their lovely smiles that can relieve any kinds of tiredness.

My sincere thanks are also to my family, **father**, **mother** and **brothers**, who did all the best to help me in my education and for their unreserved encouragement.

I would like to thank all staff members and my colleagues in Genetics department, Faculty of Agriculture, Zagazig Uni. in Egypt.

Finally, I would like to thank the Egyptian Government, Department of Missions for providing the scholarship and financial support during my study in Germany.

To all of you, Thank you very much.

Sherin Gammal El-Deen Abbas Mohamed

Bonn, 2005

Kurzfassung:

Genetische Transformation von zwei hoch-ölsäurehaltige *Helianthus annuus* L. Sorten mittels unterschiedlicher Transformationstechniken

Die stabile Transformation des hoch ölsäurehaltigen **(**HO) F_1-Hybriden cv. Capella und der HO Inzuchtlinie SWS-R2 konnte durch verschiedene Transformationsmethoden erzielt werden. Hierzu wurde zunächst für beide Genotypen ein schnelles und effizientes Regenerationssystem über direkte Embryogenese entwickelt. Dazu wurden geteilte Sprossspitzen als primäre Explantate auf zwei verschiedenen Sprossinduktionsmedien inkubiert. Innerhalb von drei Wochen konnten junge Sprosse aus den meristematischen Zonen ohne eine Kallusphase regeneriert werden. Die höchste Sprossinduktionsrate von 56-70% konnte hierbei mit SIM2 Medium beobachtet werden, bestehend aus 4,3 g/l MS Salze, 0,56 mM myo-Inositol, 0,30 µM Thiamin-HCl, 26,64 µM Glycin, 4,1 µM Nicotinsäure, 2,43 µM Pyridoxin-HCl, 3% (w/v) Saccharose, 0,44 µM BAP und 6 g/l Plant-Agar. Eine hohe Bewurzelungsrate von 60-90% konnte bei beiden Genotypen unabhängig vom verwendeten Bewurzelungsmedium erzielt werden. Die regenerierten Pflanzen konnten anschließend auf hormonfreiem Medium großgezogen werden. Aus den akklimatisierten *in vitro* Pflanzen konnten fertile Blüten und Samen erzeugt werden. Auf der Basis des bestehenden Regenerationsprotokolls konnten verschiedene Transformationstechniken wie die *Agrobacterium*-Infiltration, die kombinierte *Agrobacterium*-Infiltration durch Verwundung der Pflanzen mittels Mikroprojektilen und Glaskugeln, sowie der *Agrobacterium* Injektion in Verbindung mit der biolistischen Transformationstechnik (Gene Gun) über die Transformationsfrequenz des eingeführten *gus* Gens verglichen werden. Hierbei wurden zahlreiche Tranformationsparameter optimiert und deren Transformations – Effizienz sowohl über histochemische und fluorometrische GUS Assays als auch über Parameter der pflanzlichen Zell und Gewebekultur evaluiert. In dieser Arbeit konnte dabei gezeigt werden, dass die meisten Transformationstechniken zur erfolgreichen Transformation der hoch ölsäurehaltigen **(**HO) *Helianthus annuus* Genotypen cv. Capella und SWS-R2, ohne eine Selektion der Transformanten, herangezogen werden können. Dennoch konnte mittels PCR-Analyse zwischen den evaluierten Transformations-Methoden und den verwendeten Genotypen hinsichtlich der Transformationseffizienz Unterschiede beobachtet werden. Mittels der *Agrobacterium*-Infiltration über ballistische Mikropartikel konnten in beiden Genotypen die höchsten Transformationsraten erzielt werden. Zusätzlich konnte mit Verwendung des *mgfp5* Gens ein praktikables Marker- und Reportersystem für *Helianthus annuus* etabliert werden, welches zur sicheren Überprüfung früher Transformationsereignisse herangezogen werden kann. Jedoch führte die Verwendung des optimierten Transformationsprotokolls in Verbindung mit dem *mgfp5* Reporter Gens im Vergleich zum *gus* Reporter Gen zu einer reduzierten Transformationsrate in beiden Genotypen. Durch die histochemischen, fluorometrischen und molekularen Analysen konnte die erfolgreiche Integration des Transgens in die beiden *Helianthus* Genotypen sowie auch in deren Nachkommenschaft bestätigt werden. Eine Southern-Blot Analyse konnte darüber hinaus sowohl eine singuläre als auch multiple Integration des Transgenes in das Genom selektierter T_0 und T_1 Pflanzen aufzeigen.

Abstract:

Genetic transformation of two high oleic *Helianthus annuus* L. genotypes using different transformation methods

Stable transformation of two high oleic (HO) *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line was achieved by different transformation methods. For this, a rapid and efficient regeneration system via direct organogenesis of both genotypes has been developed. Split shoot apices explants were incubated on two different shoot induction media. Shoots from meristem adjacent tissue regenerated within 3 weeks, without a callus phase. The highest shoot induction frequency amounted to 56-70% and was observed on SIM2 medium containing Murashige and Skoog (MS) salts 4.3 g/L, myo-inositol 0.56 mM, thiamine-HCl 0.30 µM, glycine 26.6 µM, nicotinic acid 4.1 µM, pyridoxine-HCl 2.4 µM, sucrose 3% and 6-benzylaminopurine (BAP) 0.4 µM. A high rooting efficiency (60-90%) was achieved independent of genotype and rooting media. Regenerated plantlets were successfully elongated on hormone free medium. Acclimatized plantlets showed further development reaching the flowering stage and seed production. On the basis of the developed tissue culture protocol, various transformation strategies [*Agrobacterium* infiltration, combined *Agrobacterium* infiltration with wounding systems (microprojectles and glass beads), *Agrobacterium* injection and biolistic gene delivery] were compared by estimating the transformation frequency of each using the *gus* reporter gene. Several parameters affecting *Agrobacterium* infiltration method (different vacuum durations, *A. tumefaciens* strains, bacterial densities, type of explants, co-cultivation media, virulence inducers, co-cultivation durations and pre-culture periods), microprojectiles wounding (different tungsten particle sizes and particle acceleration pressures), glass bead wounding (different speeds and durations of agitation), *Agrobacterium* injection (different injection capillary sizes), biolistic gene delivery (different gold particles sizes, particle acceleration pressures, distances between macrocarrier assembly and target plate, pre-culture durations of the explant and number of bombardments per explant) were optimized. These parameters were evaluated on the basis of histochemical and fluorometric GUS activity coupled with regeneration frequency and efficiency as well as plant cell vitality. This study has demonstrated for the first time that most tested transformation methods can be successfully used to transform high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line without using selection system. However, the recorded transformation frequency (based on PCR analysis) varied among the different methods and ranged from 1.7 to 4% and from 0.9 to 4.5% in cv.capella and SWSR2 inbred line, respectively. *Agrobacterium* infiltration and biolistic gene delivery were found to be the most efficient transformation methods for cv.capella and SWSR2 inbred line, respectively. *Mgfp5* gene has proved to be a suitable reporter of early transformation events. Moreover, using the optimized transformation protocols combined with *mgfp5* gene for the transformation of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, reduced the transformation frequencies to 3.3% for both genotypes compared to *gus* gene. Histochemical, fluorometric, histological as well as molecular analysis confirmed the presence and integration of the transgene into sunflower genome and the transmission to the next generation. Southern blot analysis showed insertion of a single or multiple copies of the transgene into the genome of selected T_0 and T_1 plants.

LIST OF SYMBOLS AND ABBREVIATIONS

Abs.	Absorption
Acetosyringone	3`,5`-Dimethoxy-4`-Hydroxyacetophenon
AFLP	Amplified fragment length polymorphism
APS	Ammonium Persulphate
B5 vit.	Gamborg's vitamine mixture
BAP	6-Benzylaminopurine
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree Centigrade
CaMV	Cauliflower Mosaic Virus
CTAB	Cetyltrimethylammoniumbromide
DL-DDT	Dithiotreitol
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
EDTA	Ethylenediaminetetraacetate
g	Gram
GA3	Gibberellic acid
GFP	Green fluorescent protein
GUS	β-glucuronidase
h	Hour
hpt	Hygromycin phosphotransferase-gene
kb	killobase
l	Liter
LB	Luria Broth medium
µ	micro
m	Milli
M	Molar
MAS	Mannopinsynthase
MCT	Microcentrifuge tube
MES	2-(N-morpholino) ethanesulphonic acid

mg	Milligram (10^{-3} g)
min.	Minute
ml	Milliliter
MS	Murashige and Skoog salt mixture
MU	4-methylumbelliferone
MUG	4-Methylumbelliferyl-β-Dglucuronide
NAA	X-Naphthaleneacetic acid
NOS	Nopaline synthase
npt II	Neomycin phosphotransferase-gene
PCR	Polymerase chain reaction
PIPES	Piperazine-N, N;-bis-2-ethanesulfonic acid
Psi	Pound per square inch
PVP	Polvinylpyrrolidone
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Rotation per minute
RT	Room temperature
SDS	Sodium dodecyl-sulphate
sec.	Second
SSC	Saline sodium citrate buffer
T-DNA	Transferred DNA of *Agrobacterium*
TE	Tris EDTA
Ti-plasmid	Tumor-inducing plasmid of *Agrobacterium*
Tris	Tris-(hydroxymethyl)-aminomethan
UV	Ultraviolet light
V/V	Volume per volume
Vir	Virulence genes of *Agrobacterium*
W/V	Weight per volume
X-gluc	5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide
35S	35S promoter of CaMV

CONTENTS

INTRODUCTION

Sunflower

1- General introduction

The genus *Helianthus* belongs to *Asteraceae* family and composed of 49 species and 19 subspecies within 12 annual and 37 perennial species (Seiler, 1992; Skoric, 1993; Seiler and Rieseburg, 1997). The cultivated sunflower (*Helianthus annuus* L.) is a member of this genus. The center of sunflower origin has been identified as limited to the southwestern plains of North America, and the most species are found in the USA. Sunflower is the only plant grown world-wide on a large scale that originated and was domesticated in North America (Heiser, 1978). Natural hybridization and introgression occur between many of the species, often resulting in morphological intergradation between otherwise distinct forms. The basic chromosome number for the *Helianthus* genus is 17, and the cultivated sunflower is a diploid and open pollinating crop. Between the perennial species several variable ploidy levels of (2x, 4x and 6x) were found (Heiser, 1978; Schuster, 1993). The large variability found in the genus *Helianthus*, represents a considerable genetic pool for additional genes to improve the cultivated sunflower (Skoric, 1993). These species could provide the necessary diversity for many agronomic traits including disease and insect resistance, stress tolerance, gain in productivity and chemical composition of cultivated sunflower seeds (Seiler, 1992; Schuster, 1993; Krasnyanski and Menczel, 1995; Seiler and Rieseberg, 1997).

2-Economical importance

Initially the cultivation of sunflower (*Helianthus annuus* L.) was undertaken by America's settlers as a supplementary food. Later, sunflower was grown as a garden ornament and it was also cultivated as an ensilage crop in the late 18th century and early 19th century. The expansion of the world production of sunflower resulted primarily from development of high-oil varieties and more recently by the development of hybrids (Putt, 1978; 1997 and Schuster, 1993).

Recently, sunflower is cultivated on about 22 million hectares world-wide and with an annual seed production of 27.7 million tons (FAO, 2003). Sunflower

(*Helianthus annuus* L.) is one of the three most important annual oil-bearing crops world-wide following: soybean (*Glycine max* L.) and rapeseed (*Brassica napus* L.) crops (Weber et al., 2003). The annual sunflower oil production is 8.4 million tons world-wide and the oil has found widespread acceptance as a high quality, edible oil, rich in the unsaturated fatty acids, oleic and linoleic acids, it is also rich in vitamin E and contains about 20 % proteins. The major producing areas or countries for sunflower seeds are Argentina (3.7), European Union (4), Russian Federation (4.9), Ukraine (4.2), USA (1.2), India (1.2) and China (2 million tons). These seven areas or countries of the world produce about 76 % of the total production (FAO, 2003). France is the largest European Union producer with nearly 1.5 million tons per year. In Germany the total area comprised about 38,000 hectares with a production of 75,000 tons in 2003 (FAO, 2003), but Germany produces only about 30 % of the intern industry demand of sunflower oil. The cultivation area of the sunflower has increased significantly from 13 million hectares in 1983 to over 22 million hectares in 2003, with a small yield increase from 1.20 to 1.25 tons/hectare respectively (FAO, 2003).

In 1976, Soldatov was the first one who identified high oleic genotypes. Oil of these sunflower genotypes has 85 percent or more oleic (monounsaturated) acid. This unique oil has many special applications. High oleic sunflower oils are excellent renewable raw materials for industrial purpose. The high oxidative performance of oleic acid and its very low content of polyunsaturated fatty acids combined with low content of stearic acid make them suitable for industrial applications like cosmetics, pharmaceuticals, detergents, lubricants, metal working fluids, surfactants or for chemical syntheses. High oleic sunflower oil can also be used as food oils or deep-frying fats (Fick and Miller, 1997; Dorrell and Vick, 1997).

Biotechnology and genetic engineering

Biotechnology includes various methods and techniques of modern tissue culture, cellular and molecular biology which are relevant for plant breeding. Genetic progress is expected from those technologies both saving time and increasing genetic variation (Henry *et al*., 1994). The application of biotechnological methods to crop

improvement offers the opportunity of developing new germplasms, better adapted to the changing demands. In this way, extensive studies have been developed on sunflower biotechnology on the last decade (Kräuter *et al.,* 1991; Alibert *et al.,* 1994; Knittel *et al*., 1994; Hunold *et al*., 1995; Fick and Miller, 1997; Henn *et al.,* 1998; Müller *et al*., 2001; Cerboncini *et al.,* 2002).

1- Plant tissue culture

1.1- Tissue culture approach

The term "plant tissue culture" is commonly used to describe the *in vitro* and aseptic cultivation of any plant part on a nutrient medium (Dixon, 1987). In simple terms, plant tissue culture can be considered to involve three phases: first, the isolation of the plant (tissue) from its usual environment; second, the use of aseptic techniques to obtain clean material free of the usual bacterial, fungal, viral, and even algal contaminants, and third, the culture and maintenance of this material *in vitro* in a strictly controlled physical and chemical environment (Robert, 1999).

Eventually, plant tissue culture techniques have played an increasingly critical role in the development of gene modification and modern plant biotechnology researches (Collin and Edwards, 1998; Durante *et al*., 2002). Birch (1997) has reported that tissue culture is not a theoretical prerequisite for plant transformation, but it is employed in almost all current practical transformation systems to achieve a workable efficiency of gene transfer, selection, and regeneration of transformants.

The first step towards the use of biotechnology for sunflower improvement is the development of technology for *in vitro* culture of the pertinent *Helianthus* species or inbred lines (Robinson and Everett, 1990).

1.2- Tissue culture in sunflower

Sunflower is known as one of the most recalcitrant species for tissue culture. In 1923 Robbison and Maneval published a report describing the culture and growth of excised root tips from several plants including sunflower. It was the first reference of the response of a sterile sunflower explant in plant culture media. Sunflower root tip culture appears again in papers by White (1938), Bonner (1940) and Levine (1951). These studies defined more carefully the influence of culture media on explant

development. Definitive papers describing methods for the induction and proliferation of sunflower stem callus by media containing growth regulators were published in 1947 (De Ropp) and 1952 (Henderson *et al*.). Sunflower culture systems capable of plant regeneration have been reported since the initial observation by Sadhu (1974). The regeneration response was an isolated event; however, it offered the first example of the differentiation of entire plants from unorganized sunflower tissue. Several tissue culture variables determining the tissue culture response in sunflower such as genotype, the used explants, development stage of the explants and the time of culture initiation, culture media composition, growth conditions, acclimatization, plant establishment and fertility analysis have been reported (Gregco *et al*., 1984; Finer, 1987; Kräuter and Friedt, 1991; Knittel *et al*., 1991; Burrus *et al*., 1991; Wingender *et al*., 1996; Bidney and Scelonge, 1997; Berrios *et al*., 1999; Müller *et al*., 2001 and Dhaka and Kothari, 2002; Yordanov *et al*., 2002). Eventually, wild species of *Helianthus* have been more responsive to cell and tissue culture manipulations than cultivated *Helianthus annuus* L. (Bidney and Scelonge, 1997; Chanabé *et al*., 1991; Krasnyanski *et al*., 1992).

Sunflower has different problems with most of tissue culture protocols because these problems seriously affect the establishment and maturation of culture derived shoots in soil thereby decreasing the efficiency of the total system. The most common problems are (a) Inflorescence formation on cultured shoots, termed *in vitro* flowering, is the most frequently encountered problem. Paterson (1984) reported with adventitious shoots regenerated from half shoot tip cultures that flower buds would develop on shoots as soon as 3 weeks following culture. Normal shoots maintained for extended periods *in vitro* in an attempt to induce rooting tended to exhibit the *in vitro* flowering response. Rooting is also negatively affected by the early flowering response. Shoots that have flowered in-vitro, are very difficult to establish in soil (Lupi *et al*., 1987; Khalid *et al*., 1992b; Biasini *et al*., 1993; Alibert *et al*., 1994) and the same is true for vitreous plants. (b) Vitreous plants are the plants of a poor structural quality having a translucent, water logged appearance (Witrzens *et al*., 1988; Burrus *et al*., 1991; Knittel *et al*., 1991; Fisher *et al*., 1992). These abnormalities can reduce the number of shoots successfully grown to establish plants. Many attempts were performed to solve these problems (Witrzens *et al*., 1988; Chraibi

et al., 1992; Mayor *et al*., 2003). (c) Poor rooting establishment is a common sunflower *in vitro* problem. Rooting difficulties have been overcome by grafting small shoots on *in vitro* grown seedling rootstock or young greenhouse plants (Fischer *et al*., 1992; Krasnyanski and Menczel, 1993; Malone-Schoneberg *et al.,* 1994; Weber *et al*., 2003). Rooted or grafted sunflower shoots successfully established in the greenhouse are typically stunted and often produce a limited number of seeds. In most case, however, the subsequent generations perform normally with no residual influence from the original cultural process (Bidney and scelonge, 1997). Finally, (d) Genetic variability can also be a serious impediment for biotechnological applications. Abnormal morphological phenotypes have frequently been observed in *in vitro* regenerated sunflower plants (Power, 1987; Freyssient and Freyssient, 1988; McCann *et al*., 1988; Burrus *et al*., 1991). The callus phase is the source of somaclonal variations which occur in extended long-term callus cultures (Larkimg and Scowcroftt, 1981; Sarrafi *et al*., 1996a; Dhaka and Kothari, 2002). These variations are undesirable when improvement is targeted using transformation technology. Recently, AFLP, RFLP and RAPD analysis could be powerful tools to study the genetic stability of regenerated (Vendrame *et al*., 1999; Tang, 2001) or micropropagated plants (Goto *et al*., 1998; Watanabe *et al*., 1998).

Tissue culture in sunflower including many systems differing considerably in its results, for example the number of shoots recovered per regenerating explant is good for some systems and extremely poor for others. Therefore various strategies to establish *in-vitro* culture systems for sunflower have been attempted.

Organogenesis: Organogenesis means the complete morphological processes in which structures with the form of natural, non-autonomous organs are formed on the cultured plant material (De Klerk *et al*., 1997).The production of adventitious shoots *in vitro* is more common and easier to control than the development of somatic embryos from cultured explants. Fertile sunflower plants can be regenerate via organogenesis. This morphogenic event can be achieve either directly from the explant or indirectly from a callus induced on the explant (Charriere and Hahne, 1998). Both direct and indirect regeneration were developed from various starting materials.

Indirect plant regeneration via callus: Indirect regeneration means production of adventitious organs from a callus derived from the explant (Dixon, 1987). A variety of explants have been employed to produce callus which can in turn differentiate shoots and subsequently plantlets. Starting from Heaton's works in 1983, many authors tried to regenerate sunflower by indirect organogenesis through shoot induction on calli derived from several tissues. Among the various explants used were immature embryos (Heaton, 1983; Wilcox, 1988; Espinasse and Lay, 1989; Nedev *et al*., 1998), hypocotyls (Greco *et al*., 1984; Lupi *et al*., 1987; Prado and Bervillé, 1990) and cotyledons (Lupi *et al*., 1987 and Chraibi *et al*., 1992). However, sunflower callus culture has been used as a rapid screening system for oil content, resistance to pathogen toxins or for physiological and biochemical studies (Hartman *et al*., 1988; Masirevic *et al*., 1988).

Direct plant regeneration from cultured explant: Emergence of adventitious organs directly from the explant without an intervening a callus phase is termed "direct organogenesis" (Dixon, 1987). In other words it means production of genetically identical copies of the organ. In this procedure the explant is established on a nutrient medium containing moderate levels of auxin and cytokinins (to avoid callus production) and subsequently initiates shoot organs. Moreover, shoots multiplication is achieved through subdivision of the shooting clump and planting out in separate vessels. The first attempts to regenerate whole sunflower plants by direct organogenesis were made by Hendrickson in 1954 using shoot tips. This work was the first of several using a large diversity of genotypes and starting materials were summarized in Table 1. Paterson, in 1984, studied more than hundred genotypes and hybrid and demonstrated the requirement of cytokinin for shoot induction; its optimum concentration, however, varied with the genotype. Most of the genotypes exhibit shoot multiplication but rooting was difficult and early flowering was often observed. All the subsequent works on sunflower regeneration by direct organogenesis are closely depend on the culture condition derived by Paterson. Ceriani *et al*. (1992) proposed the use of cotyledons as potential explants. In optimized conditions, about 50 % of the studied genotypes responded positively to shooting and, in some favorable case, 50 to 90 % of the regenerated shoots managed to produce viable plants.

Table (1): Regeneration of *H. annuus* by direct organogenesis.

References	Explants	Remarks
Hendrikson, 1954	Shoot tips	-
Pall *et al*., 1981	Shoot tips	Pathogen free
Trifi *et al*., 1981	Shoot tips	
Paterson, 1984	Shoot tips	Studies on hormone requirement; rooting difficult; early flowering
Greco *et al*., 1984	Cotyledons, leaves, shoot apices, hypocotyl segments	Response different according to the explant type
Knopp and Mix, 1986	Nodes with axillary buds	-
Lupi *et al*., 1987	Shoot tips	Precocious flowering
Schettler and Mix, 1988	Seeds, cotyledons, hypocotyls, epicotyls	
Nataradja and Ganapathi, 1989	Cotyledons	High potential for direct shoot regeneration
Burrus *et al*., 1991	Protoplast	Induction regenerated plants
Knittel *et al*., 1991	Cotyledon explants from 8-day old plantlets	Precocious flowering stunted plants
Ceriani *et al*., 1992	Cotyledon explants from 3 to 6 day old plantlets	Works well with most of the studied genotype can be used for *Agrobacterium* transformation
Krasnyanski and Menczel 1993	Protoplast	Produce regenerated plants
Knittel *et al*., 1994	Shoot tip or split embryonic axes	Successful in transformation protocol
Malone-Schoneberg *et al*., 1994	Split embryonic axes	Yields Multiple shoots in an efficient manner
Laparra et al., 1995	Cotyledon, Immature embryos	Used in *Agrobacterium*-mediated protocol
Grayburn and Vick, 1995	Shoot apices	Used after wounding in transformation protocol

Burrus *et al*., 1996b	Shoot apices	-
Gürel, and Kazan, 1998	Shoot tip, cotyledon, hypocotyls, thin cell layers, cotyledonary petioles	Direct organogenesis is preferred if *Somaclonal* variation is a concern
Gürel, and Kazan, 1999	Shoot apices	-
Baker *et al*., 1999	Cotyledon	Shoot and root induction
Vischi, *et al*., 1999	Cotyledon	Used successfully in biolistic transformation
Nenova *et al*., 2000	Anther	Successes with some wild species
Müller *et al*., 2001	Hypocotyls	Used in transformation protocol
Dhaka and Kothari, 2002	Mature cotyledons	Normal, healthy elongated shoots
Hewezi *et al*., 2002	Split apices	Used in transformation protocols
Hewezi *et al*., 2003	Split embryonic axes	Regeneration restricted with the genetic pack ground of the genotype
Weber *et al*., 2003	Shoot apices	Used with macerating enzymes for transformation

Chraibi *et al*. (1992) devised an ingenious sequence of transfer from solid to liquid media which resulted in high regeneration efficiencies which are apparently independent of the genotype used.

Shoot culture: has been established in numerous species of higher plants. Plants were first recovered from cultured *Helianthus annuus* L. var. Mammoth Rossian shoot tips in 1954 (Hendrickson). The first multiplication of sunflower plants using cultured shoot tips was achieved by Pawlowski (1963) by cutting the young shoot in half and culturing the halves on Hoagland's solution containing agar. The effect of an auxin (NAA) and a cytokinin (BA) on cultured shoot tips was described by Pall *et al*. (1981). Plants developed using shoot tip culture often retains the genetic composition of the mother plant (Dixon, 1987).

Several important factors influence regeneration events and its efficiency in shoot culture. These factors such as: (1) Sunflower genotypes. Tissue culture response in sunflower has been shown to be highly genotype dependent (Gürel and Kazan, 1998; Hewezi *et al*., 2002). They tested different genotypes, hybrids and inbred lines and got strongly different responses. (2) Medium composition has been found to be an important factor in the establishment of totipotent cultures. It is well known that the relative concentration of auxin to cytokinin in the regeneration medium strongly influence shoot organogenesis in plants. The morphogenic response in sunflower is regulated by the balance between these plant growth regulators (Pugliesi *et al*., 1993a; Gürel and Kazan, 1998; Charrière *et al*., 1999). Generally, cytokinin is the main organ formation stimulator (Dixon, 1987; Espinasse *et al*., 1989; Charrière and Hahne, 1998). (3) The season in which explants are taken can often be a critical factor (Kartha, 1981). (4) Size of the explants determines the rate of survival; larger shoot tip explants invariably survive better than meristems in culture. This is due to technique skill as well as medium nutrient (Dixon, 1987). (5) Types and characteristics of explant play also a key role in the establishment of any regenerable tissue cultures and transformation success. Splitting or intact shoot apices were used in many reports (Malone-Schoneberg *et al.,* 1994; Burrus *et al*., 1996b; Hewezi *et al*., 2002; 2003; Molinier *et al*., 2002; Weber *et al*., 2003). Knittel *et al*. (1994) and Changhe *et al*. (2002) found that longitudinally splitting of shoot apices favored multiple shoot induction.

Recently, sunflower shoot apices have been reported to bear a high potential for direct shoot regeneration (Gürel and Kazan, 1998; Dhaka and Kothari, 2002; Hewezi *et al*., 2003) and it is the only explant that has been successfully used to produce transgenic offspring (Bidney *et al*., 1992; Knittel *et al*., 1994; Grayburn and Vick, 1995; Burrus *et al*., 1996b; Weber *et al*., 2003).

2- Plant genetic transformation

2.1-Transformation general introduction

Plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology (Birch, 1997). The ability to integrate DNA into an organism and alter its genotype or genetic makeup is to both basic and applied molecular biology. The first transformed plant cell lines were derived from tobacco and petunia (*Petunia hybrida* Mitchell) using kanamycin selection (Bevan *et al*., 1983a; Fraley *et al*., 1983; Herrera-Estrella *et al*., 1983). The capacity to introduce and express diverse foreign genes in plants has been extended over 120 species in at least 35 families (Birch, 1997).

- ***Transformation objectives***

Recently, many efforts were exerted in plant transformation researches because it is an efficient technique for genetic improvement. Consequently, the novel genes can be introduced to generate plant lines useful for production of materials ranging from pharmaceuticals (Haq *et al*., 1995) to biodegradable plastics (Nawrath *et al*., 1995). Transformation also provides a powerful new experimental tool, allowing direct testing of some hypotheses in plant physiology that have been exceedingly difficult to resolve using other biochemical approaches (Coruzzi and Puigdomenech, 1994).

- ***Transformation requirements***

The general requirements for a reliable and reproducible transformation system directed toward whole plant objectives are (i) a cell, tissue, or organ culture system capable of efficient and stable plant regeneration, (ii) a method of delivering plant expressible DNA into totipotent cells of the culture system; and (iii) the ability to

identify and select regenerable cells that have been stably transformed with introduced DNA (Bidney and Scelonge, 1997; Hewezi *et al*., 2002).

• ***Reporter genes***

Among different reporter genes, *gus* and *gfp* are the most widely visual reporter genes used (Jefferson *et al*., 1987; Davis and Vierstra, 1998; Taylor and Fauquet, 2002). Both β-glucuronidase (GUS) and green fluorescent protein (GFP) have been used in investigating transient, chimeric and stable gene expression (Alibert *et al*., 1999; Schrammeijer *et al*., 1990; Müller *et al*., 2001). Unlike GUS, GFP has the significant advantage that it does not require a substrate and its expression can be detected in real time in living cells by light excitation (Murray *et al*., 2004).

• ***Transformation methods***

Several techniques can introduce genes into plant cells and the most common plant transformation methods are:

i. Agrobacterium mediated transformation

Agrobacterium-mediated gene transfer is the method of choice when aiming for stable transformation in dicotyledonous species. (Hewezi *et al*., 2002). *Agrobacterium tumefaciens* is a common soil pathogen that causes crown gall disease due to its ability to transfer a section of its own DNA, known as T-DNA or "transfer DNA" during the infection process and integrate into plant genomes. The transferred DNA (T-DNA) is stably integrated into the plant genome, where its expression leads to the synthesis of plant hormones (auxins and cytokinins) which cause plant cells to grow in an unregulated manner resulting in tumor formation. These tumors then synthesize opines, a major carbon and nitrogen source for *Agrobacterium***.** *Agrobacterium* are usually classified based on the type of opines specified by the bacterial T-DNA. Depending on *Agrobacterium* ability scientists can insert interest gene into the plasmid in place of the tumor inducing genes and subsequently into the plant cell genome (Sheng and Citovsky 1996).

The process of foreign gene transfer from *Agrobacterium* into plant cells during the infection includes several sequential steps: (1) recognition of plant signals

molecules by the bacterial VirA/VirG sensor-transducer system; (2) binding of *Agrobacterium* to the host cell surface; (3) activation of the bacterial *vir* genes; (4) production of the transferable T-strand; (5) formation of the T-complex and its transport into the host plant cell complex; (6) nuclear import of the T-complex; and (7) integration of T-DNA into the plant genome (Sheng and Citovsky 1996).

Three genetic components of *Agrobacterium* are required for plant cell transformation (i) the T-DNA, which is actually transported from the bacterium to the plant cell. The T-DNA is a discrete segment of DNA located on the 200-kb Ti plasmid of *Agrobacterium*; it is delineated by two 25-bp imperfect direct repeats known as the T-DNA borders;(ii) the 35-kb virulence (*vir*) region, also located on the Ti plasmid, which is composed of seven major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*). The protein products of these genes, termed virulence (Vir) proteins, respond to the specific compounds secreted by the wounded plant to generate a copy of the T-DNA and mediated its transfer into the host cell; (iii) the suite of chromosomal virulence (*chv*) genes, located on the *Agrobacterium* chromosome (Sheng and Citovsky 1996).

Agrobacterium has significant advantages over direct gene delivery since it reduces unwanted gene silencing (Kohli *et al*., 1999), introduces one or a few copies of genes into the plant genome, high co-expression of introduced genes, defined transgene integration, relatively large segments of DNA can be transferred with little rearrangements within inserts and less fragmentation of the transgene (Hadi *et al*., 1996; Murray *et al*., 2004). In addition, *Agrobacterium* is a much more efficient transformation tool in compatible plant species compared to the particle gun protocol (Bidney *et al*., 1992).

On other hand, *Agrobacterium* mediated transformation technique prevalently use complete expression vector, sometimes leading to the integration of parts of the vector backbone sequences into the host genome along with the transgene(s). This is undesirable, as vector backbone sequences often have negative effects on transgene or endogenous gene expression and can promote transgene rearrangements. Moreover, there is concern that new replicons comprising plasmid origins of replication, bacterial antibiotic resistance genes and plant genomic DNA may escape into the environment (Prüfer, 2003). Moreover, this system does not work with all plant species, most notably the cereals (Wheeler *et al*., 1991)**.**

ii. Direct gene transfer

Direct gene transfer or vectorless gene transfer involves the transfer of DNA into protoplasts, cells, tissues or whole plants by using different chemical or physical methods.

***ii.1*- Chemical methods**

These methods relay on using different membrane-active agents such as polyethylene glycol (PEG), polyvinyl alcohol and dimethyl sulfoxide (DMSO), which destabilize membranes by creating momentarily pores allowing uptake of DNA from the surrounding solution (Machlab, 1996; David, 2001).

***ii.2*- Physical methods**

Various physical methods were used in plant transformation and the most important methods were summarized as following:

❖ ***Electroporation***

Electroporation is the process where cells are mixed with a DNA construct and then briefly exposed to pulses of high electrical voltage. The cell membrane of the host cell is penetrable thereby allowing foreign DNA to enter the host cell (Prescott *et al*., 1999). Although, transgenic rice and soybean have been produced by electroporation, success rates are low and the technique is not very reproducible (Wheeler *et al*., 1991).

❖ ***Particle bombardment***

Particle bombardment is a popular method of direct gene delivery into cells, tissues and organs. This technique uses pressurized helium to accelerate sub-cellular size microprojectiles of tungsten or gold coated with DNA (or other biological material) into cells over range of velocities necessary to optimally transform many different cell types (Bhatnagar *et al*., 2002). Since the late 1980's particle bombardment has become an efficient tool for the study of gene expression and production of stably transformed tissues and whole transgenic plants for experimental purposes and agricultural applications. Application of particle bombardment developed rapidly through the 1990's being used successfully to produce transgenic

plants in a wide range of different plant species (Taylor and Fauquet, 2002). Biolistic has the advantages of being applicable to whole plant cells in suspension or to intact or sliced plant tissues. For example, plant meristems or tissues capable of regeneration can be targeted directly. In addition, particle bombardment is a unique gene delivery approach of particular utility to monocotyledonous species (Sanford, 1988). Using biolistics, transgenic corn and soybean plants have been produced that contain heritable copies of the inserted gene. Although, this methodology is very useful for molecular genetic studies (Birch, 1997; Duchesne and Charest, 1991; Jefferson *et al*., 1987; Potrykus, 1990), it often suffers from the problem associated with direct DNA transfer method, possibly leading to gene fragmentation and silencing. Silencing results from the interactions and integration of multiple copies of the transgene (Kumpatla *et al*., 1997; Srinivasa *et al*., 2003). Several factors have been described to influence the applicability and efficiency of biolistic gene transfer. On the tissue culture side genotype (Koprek *et al.*, 1996), Plant material (Bhat *et al.*, 2001), type and age of bombarded explants (Armaleo *et al.*, 1990), culture period prior and after gene transfer (Rasco-Gaunt *et al.*, 1999) culture medium composition (Bhat *et al.*, 2001; Bhatnagar *et al*., 2002) and osmotic pre-treatment (Vain *et al.*,1993b) have been shown to be crucial. Concerning the biolistic device the applied acceleration pressure (Koprek *et al.*, 1996; Bhatnagar *et al*., 2002), the adjustable distances between rupture disc, macrocarrier, stopping screen and target plate (Bhat *et al.*, 2001; Rasco-Gaunt *et al.*, 1999), the vacuum pressure in the bombardment chamber (Bhat *et al.*, 2001; Rasco-Gaunt *et al.*, 1999), number of bombardments (Lonsdale *et al.*, 1990) as well as size and density of micro-particles (Altpeter *et al.*, 1996a; Bhat *et al.*, 2001), DNA/micro-particle mixing protocols (Perl *et al.*, 1992) and physical configuration of transforming DNA (Nandadeva *et al.*, 1999; Fu *et al.*, 2000) are factors to be optimized.

- ***Microinjection***

Microinjection is the process of directly injecting foreign DNA into the cell nucleus using ultrafine needle (Prescott *et al*. 1999). The essence of the method is a heat–induced expansion of a liquid metal alloy (galinstan, an alloy of gallium, indium and tin) in combination with silicon oil within the pipette to force probes from the

capillary tip). This technique is effective with plant protoplasts and tissues but the most obvious drawback of microinjection is damage of the cell inflicted by the penetrating glass pipette because of cellular pressure loss which often accompanied by a drastic change in the cellular ultrastructure followed by cell death. Interestingly, the narrow tip diameter (approx. 1 μm) inflicts substantially less damage than conventional capillaries and heat–induced expansion of the galinstan/ silicon oil filling allows fine control over the rate of injection. The technique is laborious, technically difficult and limited to the number of cells actually injected. However, microinjection has produced only a few transgenic plants (Wheeler *et al.*, 1991; Prüfer, 2003).

• ***Selection strategy***

Generally, successful genetic transformation requires not only efficient gene delivery but also an efficient selection system to distinguish transgenic from nontransgenic events. Different selection strategies using herbicides or antibiotics have been proposed and successfully applied in plant transformation (Wilmink and Dons, 1993). The most widely used marker genes include *nptII* and *hpt* which confer resistance to kanamycin and hygromycin, respectively. Mannose-6-phosphate isomerase (MPI) is a recently developed selectable marker (Hansen and Wright, 1999), it has been reported for successful selection of transformed sugar beet (Joersbo *et al.,* 1998). Selection requires little experimentation with the timing and concentration of selective agents to match the target tissue and gene transfer system (Birch, 1997). There are also reports of interactions between selective agent and subsequent regeneration ability (Schöpke *et al*., 1996). Thereby, transformation of some plant was achieved without using selectable genes in the process (Müller *et al*., 2001).

2.2- Sunflower transformation

Sunflower is not an amenable crop to genetic transformation. Therefore, progress in sunflower transformation has been restricted for many years by the limitations of available regeneration system and difficulty combining regeneration and transformation within the same cells (Potrykus, 1990). Despite the recent development of efficient procedures for the successful culture of somatic cells, protoplasts, and

plant regeneration (Alibert *et al*., 1994), the most efficient regeneration systems are direct, with no intervening callus phase (Power, 1987; Pugliesi *et al*., 1991). Actually, calli lose their regeneration potential rapidly. Hence, prolonged culture in the nondifferentiated state appears to be incompatible with the selection of transformed calli capable of plant regeneration (Laparra *et al*., 1995) or limited to particular genotypes (Everett *et al*., 1987; Robinson and Everett 1990). Furthermore, the chimeratic nature of the primary transformants is a major complication of the most published transformation protocols in sunflower (Durante *et al*., 2002). In addition to the problems related to gene transfer itself into the sunflower cells, attention has been paid to the use of suitable selectable markers for the recovery of transformants. Escandon and Hahne (1991) compared different selective agents and found that kanamycin is not as suitable as paromomycin or phosphinothricin as marker. Moreover, these authors demonstrated genotype/selectable marker interactions which are to be considered in transformation assays. Consequently, sunflower transformation is still far from being a routine procedure due to a poor transmission of the transgene to progenies (Molinier, *et al*., 2002)

Chimeric expression (Escandon and Hahne, 1991; Alibert *et al*., 1999; Gürel and Kazan, 1999; Hewezi *et al*., 2002) as well as stable transformation (Malone-Schoneberg *et al*., 1991, Bidney *et al*., 1992; Knittel *et al*., 1994; Müller *et al*., 2001) has been achieved by different transformation techniques.

• ***Agrobacterium tumefaciens mediated gene transfer***

Sunflower is naturally susceptible to infection with *Agrobacterium tumefaciens* (Braun, 1941) so that foreign genes have been introduced easily into sunflower tumor cells through *Agrobacterium* (Table 2). The first reported genetically engineered sunflower involved de novo regeneration of hypocotyl-induced callus, combined with *Agrobacterium* mediated gene transfer using private inbred lines (Everett *et al*., 1987). They selected kanamycin-resistant calli capable of plant regeneration after infection with an *A. tumefaciens* strain carrying the coding sequence for neomycin phosphotransferase II (NPT II). This protocol of genetically stable transformation of sunflower showed however, limited application to other sunflower genotypes (Peerbolte and Dek, 1991). Recently, using hypocotyl explants of the inbred line

HA300B, stable transformation (0.1%) were obtained after co-cultivation with *A. tumefaciens* carrying *gfp* and *nptII* genes (Müller *et al*., 2001).

Several transformation reports using various starting explants and genotypes were established and summarized in Table 2. Among the various explants used, shoot apical meristems of sunflower were dissected from seeds and co-cultivated with an *A. tumefaciens* strain harboring a binary vector carrying genes encoding β-glucuronidase (GUS) and NPTII activity (Schrammeijer *et al*., 1990). Transformation of shoot meristem cells occurred at low frequencies and chimeric expression of the two genes was observed in a few transformed shoots.

Although, the low transformation frequency of the meristematic cells limits the applicability of this procedure (Schrammeijer *et al*., 1990), technical modifications, that involved secondary culture of nodal meristems excised from transformed sector of *Agrobacterium*-infected intact meristems (Bidney *et al*., 1998: WO 98/51806), allowed the production of transgenic Sclerotinia-resistant plants of sunflower (Scelonge *et al*., 2000). In addition, it has been demonstrated that the over expression of the cytokinin-synthesizing gene *ipt* of *A. tumefaciens*, improved the induction of adventitious shoots from embryonic axes, increasing the regeneration efficiency and rate of recovery of transgenic shoots after *Agrobacterium*-mediated transformation (Molinier *et al*., 2002).

A number of factors affecting *Agrobacterium tumefaciens*-mediated transformation of sunflower have been studied. These included the physiological age of the explants (Schrammeijer *et al*., 1990), wounding explants prior to inculations (Bidney *et al*., 1992; Knittel *et al*., 1994; Grayburn and Vick, 1995), *Agrobacterium* strain/vector combinations (Bidney *et al*., 1992), co-cultivation period (Schrammeijer *et al*., 1990), hormonal composition of culture medium (Burrus *et al*., 1996b), and the type and the concentration of the selection agents (Escandon and Hahne, 1991; Pugliesi *et al*., 1993) as well as the different gene transfer methods (Moyne *et al*., 1989; Laparra *et al*., 1995).

Table (2): Gene transfer to sunflower by *Agrobacterium tumefaciens.*

References	Genotype	Gene introduced	*Agrobacterium* strain	Infected tissue and method used	Step of plant development reached
Murai *et al*., 1983		Phaseolin gene			Tumorous calli
Matzke *et al*., 1984		Zein gene	A208	Stemlets	Tumorous calli
Helmer *et al*., 1984		β-galactosidase gene from *E. coli*	A208	Hypocotyls	Tumorous calli
Goldsborough *et al*., 1986		Maiz gene	A348	Stems	Tumorous calli
Everett *et al*., 1987	Inbred lines SS410, SS415	*nptII*	LBA 288	Puncture of hypocotyls and callus induction on sections	Fertile transgenic plants
Nutter *et al*., 1987		*nptII* gene inserted into several plasmid constructs	LBA 288	Inoculation as above	Tumorous calli
Schrammeijer *et al*., 1990	*H. annuus* L. cv. Zebulon	gus, *nptII*	EHA 101	Co-cultivation with apical meristems	Chimeric transgenic plants
Hartman, 1991		Hygromycin and neomycin resistance genes	-	Hypocotyl infection, callus induction on selective media	Transgenic neomycin -resistant plants
Escandon and Hahne, 1991	Inbred lines HA300B, HA410B, RHA 274	*nptII, pat, gus*	GV3101, C58C1	Co-cultivation with hypocotyls slices	Chimeric transgenic calli
Malone-Schoneberg *et al*., 1991		*nptII*	-	Bombardment of apical metistems and co-cultivation with *Agrobacterium*	Fertile transgenic plants
Malone-Schoneberg *et al*., 1994	SMF3	*nptII*	EHA101	Bombardment of embryonic axis and co-cultivation with *Agrobacterium*	Chimeric transgenic plants
Bidney *et al*., 1992	Line SMF-3	*nptII, gus*	EHA 101	Bombardment of apical metistems and co-cultivation with *Agrobacterium*	Fertile transgenic plants
Knittel *et al*., 1992		*gus*	-	Bombardment of apical metistems and co-cultivation with *Agrobacterium*	Transgenic shoots
Pugliesi *et al*.,	Interspecific hybrid *H.*	*nptII, uidA*	GV2260	Co-cultivation with leaf disks	Transgenic plants

1993	*annuus* × *H. tuberosus*				
Knittel *et al*., 1994	Public line HA300, RHa274, RHa356	*nptII*, *uidA*	LBA 4404	Bombardment of half shoot apices and co-cultivation with *Agrobacterium*	Transgenic fertile plants
Grayburn and Vick, 1995	cv. SDB861206, hybrid Cargill 208	*gusA*	EHA105	Co-cultivation with shoot apex following glass wounding	Transgenic fertile plants
Laparra *et al*., 1995	Inbred line HA300B	*uidA*, *cat*, *nptII*	GV2260	Direct gene transfer into protoplasts, bombardment, or *Agrobacterium* co-culture with different explants	Chimeric transgenic calli and shoots
Burrus *et al*., 1996b	Inbred line HA300B, experimental inbred lines 126, 127	*uidA, nptII*	GV2260	Co-cultivation with apical segments of embryonic axess	Chimeric transgenic plants
Alibert *et al*., 1999	Inbred line HA300B	*gus*	EHA105	Co-cultivation with embryonic axis following treatment with macerating enzymes and glass wounding	Chimeric transgenic shoots
Gürel and Kazan, 1999	Commercial hybrids (Hysun 25, Hysun 36, Hysun 45 and Hyoleic 31) and public inbred lines (HA 89, HA 341, RHA 271, DL 9542, DL 9546 and DL 9548)	*gus*	LBA4404	Bombardment of shoot tips and co-cultivation with *Agrobacterium*	Chimeric transgenic shoots
Rao and Rohini, 1999	*H. annuus* L. KBSH-1	*gus, nptII*	LBA4404	Co-cultivation with two-day-old seedlings	Transgenic fertile plants
Lucas *et al.,* 2000	inbred R105	*uidA nptII,*	EHA105	Bombardment of split embryonic axes and co-cultivation with *Agrobacterium*	Transgenic fertile plants
Müller *et al*., 2001	Inbred line HA300B	*gfp*	LBA4404	Bombardment of hypocotyls explants, co-cultivation with *Agrobacterium* and sonication	Transgenic fertile plants
Molinier *et al*., 2002	Inbred line HA300B	*uidA, ipt*	GV2260	Bombardment of embryonic axes and co-cultivation with *Agrobacterium*	Transgenic shoots
Weber *et al*., 2003	Inbred line HA300B	*uidA, gfp, nptII*	GV2260	Co-cultivation with shoot tip explants using macerating enzymes and sonication	Transgenic plants

• ***Direct gene transfer to protoplasts***

Protoplast transformation has been obtained by DNA uptake mediated by chemical treatments (Moyne *et al*., 1988; Laparra *et al*., 1995) or electroporation (Burrus *et al*., 1990; Kirches *et al*., 1991; Laparra *et al*., 1995; Burrus *et al*., 1996a); nevertheless, achievement of fertile transformed plants was never reported. In sunflower protoplasts, the best results were obtained with PEG 6000 (Moyne *et al*., 1989; Laparra *et al*., 1995). Application of electroporation technique in sunflower was performed by Burrus *et al*. (1990) using a DNA construct carrying *uidA* and *nptII* genes but no transgenic plants were regenerated.

• ***Particle bombardment***

In the first report on the introduction of a foreign gene by particle bombardment of sunflower meristem explants, the regenerated plants showed *gus* expression sectors. Indicating that chimeric plants had been produced (Bidney, 1990). Transient expression of the *uidA* gene has been induced in sunflower cotyledonary explants and immature zygotic embryos at different developmental stages after microprojectile bombardment (Hunold *et al*., 1995). Small embryos of approximately 1.5-2.0 mm in diameter were the most suitable for efficient transient *gus* expression (Laparra *et al*., 1995; Hunold *et al*., 1995) and multiple shoot formation (Hunold *et al*., 1995); but, the conversion rate of transient to stable transformation was shown to be very low (Hunold *et al*., 1995). The limited success of DNA transfer into sunflower cotyledons by microprojectile bombardment is likely due to the strong cuticle (Hunold *et al*., 1995).

• ***Combination of Particle bombardment with A. tumefaciens co-cultivation***

Although, the biolistic technique allows only transient expression of foreign gene (Hunold *et al*., 1995) and /or selection of transformed chimeric shoots (Bidney, 1990; Hunold *et al*., 1995; Burrus *et al*., 1996b), its application has been decisive in sunflower transformation. With the combination of bombardment of shoot apical meristems or embryonic axes of immature embryos and the successive co-cultivation of the treated explants with *A. tumefaciens* strains, fertile transgenic plants have been obtained in several laboratories (Malone-Schoneberg *et al*., 1991, 1994; Knittel *et al*.,

1994; Lucas *et al*., 2000; Hewezi *et al*., 2001). Wounding mechanism by microprojectiles (Bidney *et al*., 1992; Malone-Schoneberg *et al*., 1994; Lucas *et al*., 2000; Hewezi *et al*., 2001) or glass beads (Grayburn and Vick, 1995) was used and reported to increase *Agrobacterium* transformation frequency. Chimeric transgenic plants were recovered after the screening of putatively transformed cells with a short (4 days) (Grayburn and Vick, 1995) or long (2-5 weeks) (Malone-Schoneberg *et al*., 1994) incubation on selective medium.

Actually, most of the previously mentioned transformation protocols suffer from low overall transformation efficiencies (Molinier, *et al*., 2002) and it is clear that all these transformation methods applied in sunflower need to be optimized before being of routine protocol (Alibert, 1994).

AIM OF THE WORK

Sunflower (*Helianthus annuus* L.) is known as one of the most recalcitrant species for tissue culture and genetic transformation. Therefore, the main objective of this research was to establish an efficient and reproducible transformation protocol for the production of transgenic high oleic sunflower (*Helianthus annuus* L.) plants. To achieve this aim several steps have been studied (Fig. 1). The pre-request of the presented investigation was to establish an efficient tissue culture system by screening diverse high oleic sunflower hybrids and inbred lines for *in-vitro* culture suitability and optimization of culture conditions for selected genotypes. On the basis of an efficient *in-vitro* culture system *Agrobacterium* infiltration, combined *Agrobacterium* infiltration with wounding systems (microprojectiles and glass beads), *Agrobacterium* injection and biolistic gene delivery strategies were compared. For each transformation method, several parameters were optimized in order to determine the most efficient transformation system. Furthermore, two reporter genes, *gus* and *gfp*, were compared. In addition, the effect of different selective agents on regeneration ability was firstly tested. Transgenic plants needed histochemical, fluorometric, histological and molecular analysis to confirm the expression, presence and integration of the transgenes in T_0 and T_1 plants.

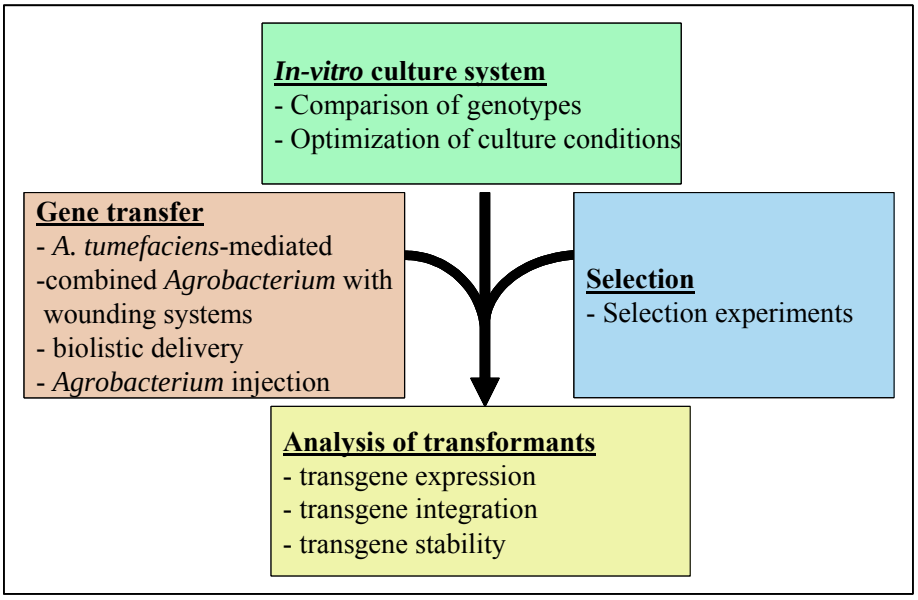

Fig. (1): Development of a reproducible transformation system for (*Helianthus annuus* L.).

MATERIAL AND METHODS

I-Material

1- Chemicals and Enzymes

Acetosyringon	Acros
Agarose	PeQGOLD
Ancymidol	SIGMA
Arylamide	MERCK
B5 vitamine	SIGMA
Bromophenol blue	SERVA
β-mercaptoehanol	SIGMA
Carbenicillin	Duchefa
Cefotaxime	Duchefa
CTAB	MERCK
DL-DTT	SIGMA
dNTP	Amersham pharmacia biotech
EcoRI (10U/µl)	Fermentas
Gentamycin	Duchefa
Hybond-N^+ membrane	Amersham pharmacia biotech
Hygromycin	Duchefa
Kanamycin	AppliChem
Light mineral oil	SIGMA
MU	SIGMA
MUG	SIGMA
Primers	MWG-biotechAG
Radioactive nucleotide [α-^{32} P]–dCTP	Amersham
Rnase	AppliChem
Spermidine	SIGMA
Streptomycin	SIGMA
Taq	Fermentas
Tris	AppliChem

X-Gluc	PeQLab

Kits

DNeasy plant Mini Kit	QIAGEN
ECL™ Kit	Amersham
Gel extraction Kit	QIA quick
Random primed hexalabeling DNA Kit	Fermentas
Restriction enzymes Kit	Fermentas

2- Equipment and instruments

Autoclave	Tuttnauer Systec ELV 3850, Holland
Automatic film developer	Optimax type TR, MS, Germany
Balance	KERN770, Germany
Binocular	Olympus, Japan
Biolistic® PDS-1000/He	Bio-Rad, USA
Camera	Kaiser RS1, Japan
Centrifuges	Eppendorf 5810R, Germany Eppendorf 5417, Germany Heraeus Sorval RC SB plus, Germany
Desiccator	Glaswerk Wertheim 365, Germany
Digital camera	NiKon E990 (3.3 MP), Japan
Electrophoresis	PeQLab, Germany
Electrophoresis power supply	Pharmacia
Fluorescence microscope	Nikon Eclipse TE 300, Japan
Garden soil (type-P)	Compo, Germany
Glass pasteurpipettes (145 mm)	Brand, Germany
Gold and tangsten particle	Bio-Rad, USA
Growth chamber	Kendro, Heraeus, Germany Snijders scientific, Germany
Hypercassette™	Amersham pharmacia biotech
Hyper-film	Kodak
Ice machine	ZIEGRA, Germany
Injection capillaries	Kwik-Fil, Germany

Injection laminar flow	Gelaire, Germany
Inverted light microscope	Olympus, Japan
Micropipette Puller	P-97, Sutter Instruments, Germany
Micropipettes	Eppendorf, Germany
Microscope	Leica microscope 10445819
Microwave	AEG, type 1301, Germany
Needle pressure delivery system	Eppendorf, Germany
Oven	Biometer OV3, Germany
particle delivery system	Bio-Rad, USA
PCR thermocycler	TECHNE, GENIUS, England
pH meter	Inolab, Germany
Photoaparat for agarose gels	Camera and transilluminator (VI / BER Lourmat V0292-97) Videoprinter MITSUBISHI P91,Japan
Phototron	YORK international, USA
Portable fluorometer	PAM 2000,Waltz, Effeltrich, Germany
Shaker	Certomat HK, KS125 basic, Germany
Spectro fluorometer	Fluoro-Max, Spex Industries, USA
Spectrophotometer	UV mini 1240, UV-VIS, USA
Stirrer	Heidolph MR 3000, Germany
Strile bank	Holten Lamin Air, Danemark
Vacuum pump	Diaphragm, type ME 4R, Germany
Vortex apparatus	REAX TOP, Heidolph, Germany
Waterbath	KÖTTERMAN, Germany

3- Plant material.

The experiments of this investigation were carried out on seeds of five high oleic sunflower (*Helianthus annuus* L.) genotypes, three hybrids, cv.capella, cv.prolic-204 and cv.orbaril and two inbred lines SWSR1 and SWSR2. Seeds were kindly provided by different sources (Table 3).

Table (3): Seed sources and breeder names of high oleic *Helianthus annuus* L.

Genotype	Seed source	Breeder
cv.capella	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)
Prolic-204	Rustica Saatzucht GmbH Hamburg (Germany)	Rustica prograin Genetique(Germany)
Orbaril	Pioneer Hi-Bred Northern Europe GmbH	Pioneer Semences Aussonne, France
SWSR1	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)
SWSR2	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)	Südwestdeutsche Saatzucht Dr. Hans Ralf Späth Rastatt (Germany)

Seeds were selected for uniformity of size and color before use.

II- Methods

1- Surface sterilization and germination of seeds

Seeds were surface sterilized for 1 min. in 70 % (v/v) ethanol followed by 6 % (w/v) sodium hypochlorite solution containing one drop of Tween 20 for one hour and finally washed three times in sterilized distilled water. The sterilized seeds were germinated on modified MS (Murashige and Skoog, 1962) medium containing:

½ MS salts	2.15 g/l
Sucrose	2 % (w/v)
MES	3.2 mM

The medium was solidified with 7.5 g/l phytoagar. The pH was adjusted to 5.7 ± 0.1 with NaOH (1M) prior to addition of the gelling agent and autoclaving at 121°C, 1.2-1.3 Kg/cm^2 pressure for 20 min. The sterilized seeds were aseptically sown in 750 ml Weck glasses containing 100 ml of geminating medium. The glasses were kept in a growth chamber at 25 ± 1°C and a light period of 12 h (115 µ E $m^{-2}s^{-1}$) for 10 days.

The germination efficiency of 300 seeds was scored (Number of germinated seeds/ total number of cultured seeds *100).

2- Direct regeneration

2.1- Media

Two different shoot induction media (SIM) were tested (Table 4). The pH of all the media was adjusted to 5.7 ± 0.1 with NaOH (1M) prior to addition of the gelling agent and autoclaving at 121°C, 1.2-1.3 Kg/cm^2 pressure for 20 min. The media were poured in Petri dishes (9 cm in diameter), sealed with parafilm and kept at RT for use.

Table (4): Shoot induction media (SIM) tested.

Component	SIM1	SIM2
MS-salt (g/L)	4.3	4.3
Sucrose (%)	1	3
Glycin (µM)	-	26.64
Myo-inositol (mM)	-	0.56
Thiamin-HCl (µM)	-	0.30
Nicotinic acid (µM)	-	4.1
Pyridoxine-HCl (µM)	-	2.43
Agar-agar (g/L)	8	-
Plant agar (g/L)	-	6
BAP (µM)	0.44	0.44

SIM1, Zimmermann, (1999)

SIM2, Müller *et al*., (2001)

Plant growth regulators were generally sterile filtered through 0.2 µm filter and added to the medium after autoclaving.

2.2- Explant preparation and regeneration on different shoot induction media

After appearance of the first leaf, aseptic shoot apices (4-5 mm length) were excised and two types of shoot apices were prepared (1) intact shoot apices with a complete meristematic dome and (2) split shoot apices, shoot apices bisected longitudinally in two halves with the cut passing through the apical meristem and between the remainders of leaf primordial according to Malone-Schoneberg *et al*.,

(1994), Fig.2. The meristem adjacent tissue was cultivated in Petri dishes containing 30-40 ml of different shoot induction media (SIM). Explants on SIM were incubated in growth chamber at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}). After three weeks of shoot induction the explants were sub-cultured on fresh SIM2. The experiments were laid out as a completely randomized design and each treatment was based on 200 explants (4 replicates with 50 explants/each).

❖ ***Evaluation of regeneration***

After 25-27 days of sub-culturing, regeneration frequency was recorded as a mean percentage of explants with shoot induction (number of regenerated explants /total number of explants *100), % of shoots with hyperhydratation, % of shoots with callus formation, time of shoot primordial initiation in days, number of regenerated shoots per explant, and scoring system was used to evaluate the regeneration efficiency according to Sharon and Lineberger, (1983) as following:

Negative result	= 1
Below average	= 2
Average	= 3
Above average	= 4
Excellent	= 5

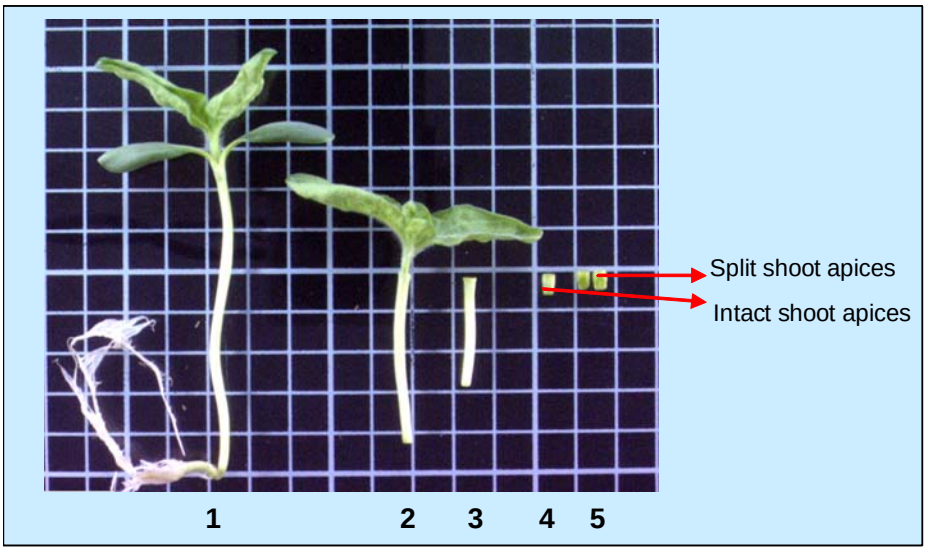

Fig. (2): Preparation steps of intact and split shoot apices

3- Shoot elongation

3.1- Media

Two different shoot elongation media (SEM) were tested (Table 5). The media were prepared as previously described in (II.2.1), poured in Weck glasses (140 ml) and kept at RT for use.

Table (5): Different shoot elongation media (SEM) used.

Component	SEM1	SEM2
MS-salt (g/L)	4.3	2.15
Sucrose (%)	2	2
Myo-inositol (mM)	0.56	0.28
$AgNO_3$ (µM)	-	5.8
MES mM	-	2.8
Thiamin-HCl (µM)	0.30	-
Nicotinic acid (µM)	4.1	-
Pyridoxine-HCl (µM)	2.43	-
Glycin (µM)	26.64	-
PH	5.7	5.6
B5-vitamine(1000x)ml	-	0.5
Agar-agar (g/L)	7.5	-
Phytagel (g/L)	-	4

SEM1 hormone free medium, Murashige and Skoog, (1962)

SEM2, Henn, (1998)

3.2- Shoot culture on different shoot elongation media

Regenerated shoots on SIM2 of cv.capella and SWSR2 inbred line longer than 1.5 cm were divided to two groups: one group cultured directly on different root induction media (II.4.1) and the other group was transferred to Weck glasses (160 ml) containing 50-60 ml of different SEM media (Table 5) as following: SEM1 hormone free and SEM2 with 4.4 µM BAP, 0.54 µM NAA and 0.29 or 0.58 or 1.45 µM GA3.

The glasses were kept in a growth chamber at 25 ± 1°C and a light period of 12 h (115 µ E $m^{-2}s^{-1}$).

❖ ***Evaluation of elongation***

Shoots were scored for elongation as following:

No elongation = +

Shoot length 1-2 cm = ++

Shoot length 2-3 cm = +++

Shoot length 3-4 cm = ++++

Shoot length > 4 cm = +++++

This scoring system is regardless the length of the regenerated shoots.

4- Root induction

4.1- Media

The different root induction media (RIM) used were summarized in table 6.

Table (6): Evaluation of different root induction media (RIM).

Component	RIM1	RIM2
MS-salt (g/L)	2.15	2.3
Sucrose (%)	1	2
Myo-inositol (mM)	0.28	0.28
$AgNO_3$ (µM)	-	5.8
MES (mM)	-	2.8
Thiamin-HCl (µM)	0.15	-
Caseine hydrolysat (g/l)	-	2
Glycin (µM)	-	13.3
PH	5.7	5.6
Phytagel (g/L)	4	4
B5-vitamine(1000x)ml	-	0.5
Ancymidol (µM)	1.95	-
NAA (µM)	0.54	-

RIM1, Fiore *et al*., (1997)

RIM2, Henn, (1998)

4.2- Shoot preparation and culture on different root induction media

The well developed shoots of cv.capella and SWSR2 inbred line from both groups (elongated and non-elongated shoots) were excised and transversely cut at the base of the hypocotyls before culturing on the different root induction media (Table 6). Before transferring the shoots to RIM2, the lower parts were dipped for one sec. in 5.3 mM NAA. Three replicates of thirty shoots were used for each treatment. Shoots on the rooting media were incubated in growth chamber at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}).

❖ ***Evaluation of rooting***

Rooting was evaluated as rooting frequency which calculated as a mean percentage of shoot with root induction (number of rooted shoots /total number of cultured shoots *100), root primordial initiation in days, root density (number of roots / regenerated shoot) and root description (length and thickness).

5- Shoot elongation and root development

5.1- Media

Two different shoot elongation and root development media (SER) were used to elongate the regenerated shoots and in the same time development the rooting system (Table 7).

Table (7): Test of different shoot elongation and root development media

Component	SER1	SER2
MS salt (g/L)	2.15	4.3
Sucrose (%)	1	3
Myo-inositol (mM)	0.56	0.56
Thiamin-HCl (µM)	0.30	1.19
Nicotinic acid (µM)	4.1	-
Pyridoxine-HCl (µM)	2.43	-
Glycin (µM)	26.64	-
PH	5.7	5.7
Phytagel (g/L)	4	4
GA3 (µM)	2.89	-

SER1**,** modified Henn, (1998) and SER2, Fiore *et al*., (1997)

5.2- Culture of plantlets on different shoot elongation and root development media

The plantlets of cv.capella and SWSR2 inbred line, with initial roots which were regenerated on SIM2 and directly rooted on RIM1, were transferred to different shoot elongation and root development media (Table 7). The plantlets were grown in a chamber at 25±1°C and a light period of 15 h (147 µ E m^{-2} s^{-1}) and evaluated for *in vitro* flowering, shoot elongation in days and scoring system was used for elongation as mentioned in II.3.2.

6- Plantlets acclimatization and evaluation

Plantlets with well-developed roots were carefully removed from the tubes and washed to remove the agar. Then transferred to small pots containing a mixture (1:1) of soil and garden-soil type-P and cultured in a growth chamber at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}), regularly irrigated until flowering, synthetic hybridization and seed production. Plantlets were evaluated for surviving by soil transfer (%), time to flowering (weeks), number of seeds/ head and mean of plant height (cm).

7- Transformation of shoot apices using *gus* gene

For all the transformation experiments, split shoot apices of high oleic sunflower (cv.capella hybrid, and SWSR2 inbred line), SIM2 (II.2.1), RIM1 (II.4.1), SER2 (II.5.1) media, and acclimatization protocol (II.6) were used.

7.1- *Agrobacteria* elimination

Two different antibiotics, cefotaxime and carbincillin, which are widely used antibiotic for *Agrobacterium* elimination after co-cultivation period in transformation experiments, were used to study their effect on shoot induction frequency and efficiency.

❖ ***Effect of different cefotaxime and carbincillin concentrations on plant regeneration***

An efficient concentration of cefotaxime and carbincillin for *Agrobacterium* elimination, without affecting on the regeneration efficiency, was determined by

culturing non-transformed (control) explants on SIM2 supplemented with different concentrations of cefotaxime (0, 125, 250, 500 and 1,000 mg/l) or carbincillin (0, 25, 50, 100 and 200 mg/l). The antibiotic was filter-sterilized prior to the addition to the regeneration medium. The explants were transferred to the same fresh medium containing the same level of antibiotic every three weeks for a total of six-seven weeks then the regeneration percentage and efficiency were scored as previously mentioned in (II.2.2). These experiments were based on four replicates of forty explants for each treatment.

7.2- Sensitivity to selective antibiotics

To identify the efficient concentration of kanamycin and hygromycin for selection of transformed plants, a pre-experiment was carried out to test the effect of different antibiotics concentrations on shoot regeneration. Non-transformed explants were prepared as previously described and cultured on SIM2 containing 250 mg/l cefotaxime and 0, 12.5, 25, 50, 100, and 200 mg/l kanamycin or 0, 2.5, 5, 10, 20 and 40 mg/l hygromycin. The antibiotics were filter-sterilized before adding to the regeneration medium. After six-seven weeks the regeneration percentage and efficiency were scored as in section (II.2.2). These experiments were based on five replicates of thirty explants for each treatment.

7.3- Transformation methods

7.3.1- *Agrobacterium* infiltration method

- ***Bacterial strains and vectors***

Four *Agrobacterium tumefaciens* strains were used from different opine types (Table 8)

Table (8): *Agrobacterium tumefacien* strains and plasmids which used in this study.

Bacterial strain	Helper plasmid	Antibiotic resistance	Opine type	Reference	Binary plasmid
LBA4404	pAL4404	Streptomycin	Octopine	Hoekema *et al*., 1983	pBI121
C58	pTiC58	Streptomycin	Nopaline	Han *et al*., 2000	pAM194
GV3101	pMP90	Gentamycin	Nopaline	Koncz and Schell, 1986	pBI121
EHA101	pTi-EHA101	Kanamycin	Agropine	Hood *et al*., 1986	pIBGus

All bacterial strains containing ß-glucuronidase (*gus*) reporter gene under the transcriptional control of cauliflower mosaic virus 35S promoter and the selectable marker neomycin phosphotransferase (*nptII)* gene under the control of nopaline synthase (nos) promoter except C58, which carries *gus* gene under the control of mannopine synthase (mas) promoter.

For all the transformation experiments, except in testing effect of different *Agrobacterium tumefaciens* strains, LBA4404 strain was used with cv.capella and GV3101 strain with SWSR2 inbred line. Both strains are harboring the plasmid, pBI121 (Fig. 3) (Chen *et al*., 2003).

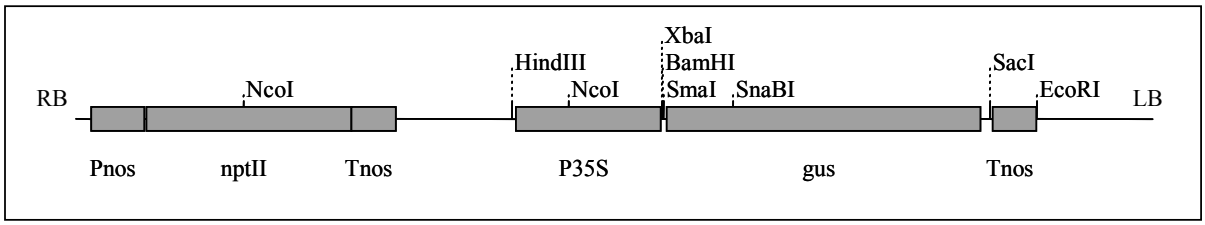

Fig. (3): Schematic representation of the T-DNA of the plasmid pBI121 used for transformation of two high oleic *H. annuus* L. genotypes (cv.capella and SWSR2 inbred line) containing the *gus* and *nptII* genes under different constitutive promoters.

❖ ***Agrobacterium culture and maintenance***

Selection of *Agrobacterium* was done on YEB medium plates (Sambrook *et al*., 1989) containing:

Beef extraction	5 gm/l
Yeast extraction	1 gm/l
Tryton-pepton	5 gm/l
Sucrose	5 gm/l

The medium was solidified with 15 g/l bacto-agar and 5 ml/l of 1M $MgSO_4$, filter-sterilized, was added after autoclaving, then supplied with appropriate antibiotics (Table 8). For all the transformation experiments the *Agrobacteria* were maintained by sub-culturing single colonies on fresh plate every 4-6 weeks. *Agrobacteria* were cultured overnight on YEB liquid medium at 28 °C with continuous shaking at 200 rpm under appropriate antibiotic. Selected cells from

overnight cultured were centrifuged at 4,000 rpm for 15 min. at room temperature, washed once in one volume MS medium (Murashige and skoog, 1962) and centrifuged under the same condition, then re-suspend once in either MS or YEB medium to achieve the required OD_{600}, with or without the addition of different virulence inducers. If applied, the bacterial suspension was incubated for 2 h. with the respective inducer prior to use.

a- Effect of vacuum duration on split shoot apices transformation

These experiments were designed to investigate the effect of different vacuum durations on split shoot apices transformation and cell vitality, represented by fluorometric and histochemical Gus activity, as well as regeneration percentage, efficiency and plant cell vitality. The *Agrobacterium* used was GV3101 and the vacuum system consisted of a vacuum pump to which a desiccator was attached. Sterile plastic eppendorf tube containing explants immersed in inoculum (*Agrobacterium* suspension with an OD_{600} of 1.2) was placed in the desiccator and the vacuum applied at 150 mbar for a period (0, 30, 60, 120, 240 and 480 sec.), the pump was switched off and then rapidly ventilated. The infiltration procedure was repeated two times. Inoculum was removed and the explants blotted dry on sterile Whatmann 3MM filter paper, then cultivated in Petri dishes containing SIM2 at 25 ±1°C and a light period of 12 h (115 μ E m^{-2} s^{-1}). Following co-cultivation, explants were washed twice in liquid SIM2 containing 250 mg/l cefotaxime, blotted on sterile Whatmann 3MM filter paper and cultured on solid SIM2 containing the same concentration of the antibiotic to suppress the growth of the *Agrobacterium*. Dishes were sealed with parafilm and cultured under the same previously described conditions. This experiment was based on three replicates of thirty explants for each treatment.

b- Optimization of parameters enhancing transformation efficiency.

The transformation parameters were optimized for split shoot apices using the bacterial strains (GV3101 with SWSR2 and LBA4404 with cv. capella). Parameters were tested, one at a time in a sequential order. The following parameters were tested in the order stated; bacterial strains (GV3101, LBA4404, C58 and EHA101) (Table 8), density of bacterial culture (OD_{600} 0.5, 1.0, 1.5 and 2.0), explant characteristics

(split and intact shoot apices), co-cultivation media (MS and YEB), virulence inducer (200 µM acetosyringone and 100 µM coniferyl alcohol), co-cultivation duration (2 and 3 days) and pre-culture (0, 1, 2 and 3 days). These Parameters were evaluated on the basis of histochemical and fluorometric Gus activity coupled with regeneration frequency and efficiency and plant cell vitality.

❖ ***Co-cultivation of explants with Agrobacteria***

Explants were prepared as previously described in (II.2.2). For each transformation experiment 60-100 explants were divided into groups. Each group was immersed, directly or after 1, 2 or 3 days of pre-culture, in 2 ml *Agrobacterium* suspension for 30 min. and transferred to vacuum infiltration flask (desiccator). Vacuum was applied at 150 mbar for 1 min., then rapidly ventilated. The infiltration procedure was repeated two times. *Agrobacterium tumefaciens* suspension was removed and the explants were dried using Whatmann 3MM filter paper, then cultivated in Petri dishes contain shoot induction medium (SIM2). After co-cultivation period the explants were washed twice in liquid SIM2 containing 250 mg/l cefotaxime, blotted on sterile Whatmann 3 MM filter paper and cultured on solid SIM2 supplemented with 250 mg/l cefotaxime to eliminate the *Agrobacterium* without using selection agents. Dishes were sealed with parafilm and cultured under the same previously described conditions. After three weeks of shoot induction the explants were sub-cultured on fresh SIM2 with the same antibiotic concentration.

For testing the first parameter, shoot split apices were pre-cultured on shoot induction medium (SIM2) for 2 days, transformed using different bacterial strains re-suspended in MS medium at optical density (OD_{600} 1.2) without virulence inducer and co-cultivation 2 days on SIM2 at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}).

All the resulting optimal parameters were applied in final representative transformation experiments using split shoot apices of cv.capella and SWSR2 inbred line and these experiments were performed on 100 explants for each genotype and the transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated explants.

7.3.2- Using different wounding strategies and *Agrobacterium* infiltration

In these transformation experiments, microprojectiles and glass beads were used as two different wounding strategies combined with *Agrobacterium* infiltration method. The experiments were laid out as a complete randomized design and each treatment was based on four replicates of forty explants each. Evaluation of these conditions was based on histochemical and fluorometric Gus activity coupled with regeneration percentage and efficiency and plant cell vitality.

a- Using particle bombardment for wounding the tissue

❖ ***Preparation of the tungsten particles***

Preparation of the tungsten particle was performed according to the method of Sanford *et al*., (1993) for 120 bombardments using 500 µg of the microcarrier. For each preparation 30 mg of microparticles were weighted out into 1.5 ml MCT, 1 ml of 70 % (v/v) ethanol was added to it. The MCT was vortexed vigorously for 3-5 min. and the particles were remained soaked in 70 % (v/v) ethanol for 15 min. The micro-particles were pelleted by centrifugation for 5 sec. at 14,000 rpm. The microparticles were washed three times by adding 1 ml sterile water, vortexing vigorously for 1 min. allowing the particles to settle for 1 min. and pelleting the microparticles by briefly centrifugation. After the third wash, the particles were suspend in 500 µl of sterile 50 % (v/v) glycerol and stored at -20 ºC at a final concentration of 60 mg/ml. The microparticles suspension was thawed when required and vortexed vigorously for 5 min. to resuspend the particles. 50 µl aliquots (3 mg) were taken in 1.5 ml MCT. Directly before using, the tungsten aliquots were centrifuged for 5 sec. at 14,000 rpm, the liquid was removed and replaced by 140 µl (100 %) ethanol. Tungsten aliquots were re-vortexed for 1 min., the tungsten particles were pelleted by centrifugation for 5 sec. at 14,000 rpm and 48 µl (100 %) ethanol were added. Each tube was vortexed at low speed for 2-3 sec. and 6 µl were taken for each macrocarrier.

a.a- Assessment of different physical factors

Forty split shoot apices were prepared as previously described in (II.2.2), grouped in center of 4 cm Petri dishes on 2 % (w/v) autoclaved agarose gel (the cut surface facing up), bombarded according to Bidney *et al*., 1992 using Biolistic® PDS-

1000/He particle delivery system to induce wounds on the explants using different uncoated particle sizes (0.4 and 0.7 µm) and different particle acceleration pressures (0, 450, 900, 1550 and 1800 psi) at a distance of 6 cm between the macrocarrier and target plate. Explants were directly imbibed in *Agrobacterium* suspension with an OD_{600} of 1.0. After 30 min. explants were transferred to vacuum infiltration flask and vacuum was applied as described in (II.7.3.1.b). *Agrobacterium tumefaciens* suspension was removed and the explants were dried using Whatmann 3 MM filter paper, then cultivated in Petri dishes containing SIM2 and incubated at 25 ±1 °C and a light period of 12 h (115 µ E m^{-2} s^{-1}). Three days after co-cultivation, explants were washed twice in liquid SIM2 containing 250 mg/l cefotaxime, blotted on sterile Whatmann 3MM filter paper and cultured on solid SIM2 containing the same concentration of the antibiotic without using selection agents. Dishes were sealed with parafilm and cultured under the same previous conditions. After three weeks of shoot induction the explants were sub-cultured on fresh SIM2 with the same antibiotic concentration.

All the resulting optimal parameters were applied in final representative transformation experiments using 100 split shoot apices of cv.capella and 90 of SWSR2 inbred line and the transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated explants.

b- Using glass beads for wounding the tissue

b.a- Optimization of different wounding parameters

Explants were prepared as previously described in (II.2.2), introduced into 2 ml autoclaved eppendorf tubes containing 0.5 g autoclaved glass beads (425-600 µm diameter) and 200 µl of *Agrobacterium tumefaciens* suspension (OD_{600} value 2.0) + 200 µM acetosyringone. Tubes were shaken using REAX TOP vortex apparatus at different speeds (0, 1,000 and 2,000 rpm) and durations (0, 10, 20, 40, 60 sec). Explants were transferred to new eppendorf tubes and 2 ml of *Agrobacterium* suspension with an OD_{600} of 1.0 were added to each. After 30 min., explants were infiltrated for 1 min. two times at 150 mbar, then removed individually from the bacterial suspension, dried on filter paper, cultured in 9 cm Petri dishes containing

SIM2 and incubated at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}). After three days, explants were washed twice in liquid SIM2 containing 250 mg/l cefotaxime, blotted on sterile Whatmann 3 MM filter paper and transferred to solid SIM2 supplemented with 250 mg/l cefotaxime without using selection agents and cultured under the same conditions. After three weeks of shoot induction the explants were sub-cultured on fresh SIM2 with the same antibiotic concentration.

All the resulting optimal parameters were applied in final representative transformation experiments using 112 split shoot apices of cv.capella and 110 of SWSR2 inbred line and the transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated explants.

7.3.3- *Agrobacterium* injection

All the injection procedures were performed under the laminar flow using inverted light stereo microscope.

- ***Preparation of Agrobacterium suspension***

Single colony of each *Agrobacterium* strain, LBA4404 and GV3101 was selected and cultured as previously described in section (II.7.3.1). The OD_{600} of the *Agrobacterium* suspension was adjusted to value 1.0 and incubated with 200 µm acetosyringone for 2 h at RT before use.

- ***Explant sterilization and preparation for injection***

Explants were sterilized and prepared as described in section (II.2.2), placed individually on 6 cm Petri dishs containing 2 % (w/v) agarose and the cut surface was positioned for injection needle tip

- ***Preparation of the loading capillary***

The glass Pasteur pipettes were drawn or pulled to a fine point by manual heat–induced expansion, autoclaved for 20 min. at 121 ºC and 1.2-1.3 kg/cm^2 pressure and finally used for filling the injection needle.

❖ ***Preparation of the injection needle and loading***

Borosilicate glass capillaries were pulled with a method based on heat-induced expansion of galinstan (aliquid metal alloy of gallium, indium, and tin) within a glass syringe to expel samples through an ultrafine tip diameter using a micropipette puller. The needles were pulled to a tip outer diameter of (7-9 and 25-27 μm) and sterilized for 30 min under UV lamp prior to use. *Agrobacterium* suspension was loaded into the needle by backfilling as following: one-third *Agrobacterium* suspension, one-third air and one-third *Agrobacterium* suspension.

❖ ***Mount the injection needle in the microinstrument holder and injection***

Firstly the air which may be trapped in the instrument holder was expelled by turning the micrometer cell tram oil until oil drips out the front. Then, the cap of the holder was loosed, the injection needle (containing the *Agrobacterium* suspension) gently pushed in the instrument collar and finally the cap gently screwed on. Both the instrument holder and injection needle were mounted onto the micromanipulator stand for injection. Explants were pierced more than one time in the meristematic region aiming meristematic cells and the tip of the needle remains inside, while the suspension is expelled from the needle using the hydraulic fluid of the light mineral oil as a needle pressure delivery system with adjusting the micrometer so that there is no excess pressure or suction (Fig. 4). Thirty injected explants (ten explants per plate for each treatment) were co-cultivated for three days on SIM2. Following co-cultivation, explants were washed twice in liquid SIM2 containing 250 mg/l cefotaxime, blotted on sterile Whatmann 3MM filter paper and cultured on solid SIM2 containing the same concentration of the antibiotic to suppress the growth of the *Agrobacterium* and without using selection agents. Dishes were sealed with parafilm and cultured under the same previously conditions. After three weeks of shoot induction the explants were sub-cultured on fresh SIM2 with the same antibiotic concentration.

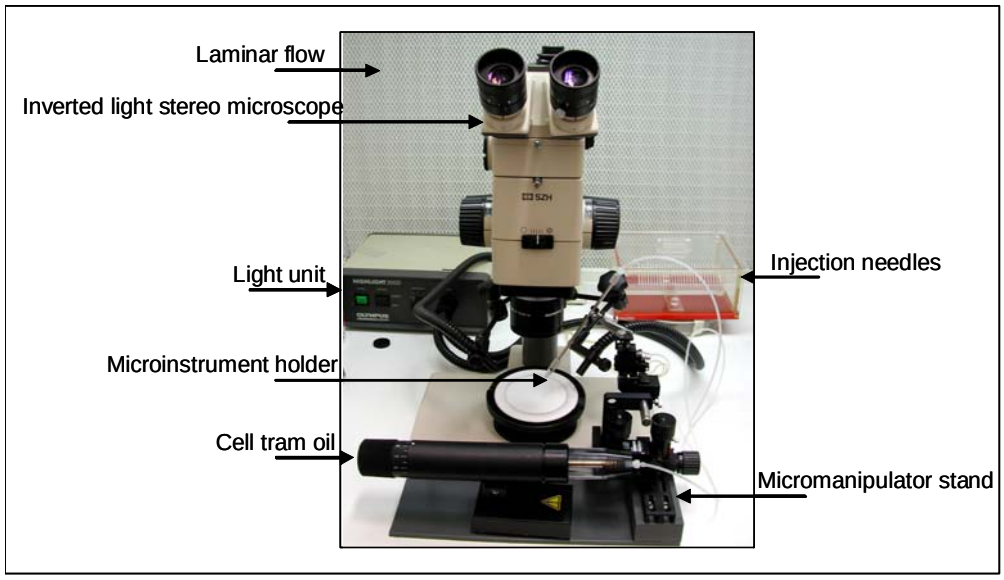

Fig. (4): *Agrobacterium* injection apparatus.

7.3.4- Biolistic gene transfer

❖ ***Mini plasmid isolation from E. coli***

Mini preparation of plasmid DNA from *E. coli* DH5α carrying the plasmid pBI121or pCAMBIA1302, was performed as described by Maniatis *et al*., (1982) using the alkaline lysis method. The bacteria from 5 ml LB medium (Sambrook *et al*., 1989) were collected by centrifugation and re-suspended in 200 µl of 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA, then 400 µl of 0.2 NaOH, 1% (w/v) SDS solution were added. The contents were mixed and incubated in RT. for 5 min. Then 300 µl of cold solution of 3 M potassium acetate (pH 0.5) and 1.8 M formic acid were added, shaken vigorously and further incubated for another 5 min. on ice, centrifuged for 5 min. at 14,000 rpm at RT. The supernatant was transferred to a fresh tube and 400 µl isopropanol were added and well mixed. The mixture was centrifuged for 30 min. at 14,000 rpm at RT. and the pellet was washed with 70% (v/v) ethanol. Finally the pellet was dried and re-suspend in 20-30 µl TE buffer containing RNase (10 µg/µl final concentration). The DNA was quantified and checked for its quality by gel electrophorasis.

❖ ***Preparation of the gold particle and coating with DNA***

Preparation of the gold particles was performed as described in section (II.7.3.2.a) and directly before using the gold, aliquots were divided to 50 µl (3 mg) in 1.5 MCT and while vortexing continuously (for uniform DNA precipitation onto microcarriers) and the following were added sequentially: 5 µl DNA (1 µg/ µl), 50 µl 2.5 M Ca Cl_2 and 20 µl 0.1 M spermidine. Contents were vortexed for 5-6 min., microcarriers allowed to settle for 1 min, pelleted by spinning for 2 sec at 14,000 rpm and the liquid removed and replaced by 140 µl of 70 % (v/v) ethanol for washing. The first washing was followed by washing with 100 % ethanol and finally particles were resuspend in 48 µl of 100 % ethanol. These coated particles were kept at 4 ºC and used within 1 h of preparation and 6 µl of the coated particles suspension was loaded on the macrocarrier membrane which was allowed to dry for 10 min. prior to use.

a- Evaluation of different physical and biological parameters

A set of various physical and biological parameters, that support the introduction of DNA with minimal tissue damage or interference with the regeneration potential, were optimized in a single or multifactorial way using pBI121 coated gold particles for cv.capella hybrid and SWSR2 inbred line. Split shoot apices prepared as previously described in (II.2.2), grouped in center of 4 cm Petri dishes on 2 % (w/v) autoclaved agarose gel (the cut surface facing up). The tested parameters including different gold particles size (1 and 1.6 µm), particle acceleration pressures (0, 450, 900, 1550 and 1800 psi), distance between macrocarrier assembly and target plate (6 and 9 cm), pre-culture of the explant (0, 1 and 2 days) and number of bombardments / explant (1 and 2 shot). The bombardment was performed according to Sanford *et al*., (1993) using Biolistic® PDS-1000/He particle delivery system. Bombarded explants were cultured on SIM2 at 25 ±1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}). After three weeks of shoot induction, explants were sub-cultured on fresh SIM2 and incubated under the same conditions. Evaluation of these parameters was based on histochemical and fluorometric Gus activity coupled with regeneration percentage and efficiency and plant cell vitality. These experiments were laid out as a complete randomized design and each treatment was based on four replicates of forty explants. All the resulting optimal parameters were applied in final representative

transformation experiments using 95 split shoot apices of cv.capella and 110 of SWSR2 inbred line and the transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated explants.

7.4- Measurement of plant cell vitality

Random treated regenerated shoots were chosen to measure the vitality by using PAM 2000 fluorometer [pulse-amplitude modulated system], Waltz, Effeltrich, Germany, (Schreiber and Bilger, 1993). Vitality was measured as a yield, which represents the essence of fluorescence quenching analysis by the saturation pulse method and calculated according to the equation

$Y = (Fm' - Ft): Fm'$

Fm´ = the parameter which represents the measured fluorescence yield at any given time.

Ft = the parameter defined as the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample.

7.5- Stable transformation

In these final transformation experiments of *gus* gene 120 shoot apices of cv.capella were transformed using the previous optimized *Agrobacterium* infiltration protocol (OD_{600} =1, using split shoot apices, MS co-cultivation medium, addition of 200 µM acetosyringone, 3 days co-cultivation duration and 3 days pre-culture period) whereas 125 shoot apices of SWSR2 inbred line were transformed using the previous optimized particle bombardment protocol (1.6 µm gold particle, 6 cm target distance, 1550 psi, 2 shot per plate and pre-culture for 1 day). The transformed plantlets were further developed until seed production as described before in II.6.

8- Transformation of shoot apices using *gfp* gene

- ***Bacterial strain /binary plasmid***

Agrobacterium tumefaciens LBA4404 strain (opine group) carring the pCAMBIA 1302 plasmid was used in the currently transformation experiment. This bacterial strain harboring the *mgfp5* gene (green fluorescent protein) and the selectable

marker hygromycin phosphotransferase (*hpt)* genes under the transcriptional control of cauliflower mosaic virus 35S promoter (Fig. 5).

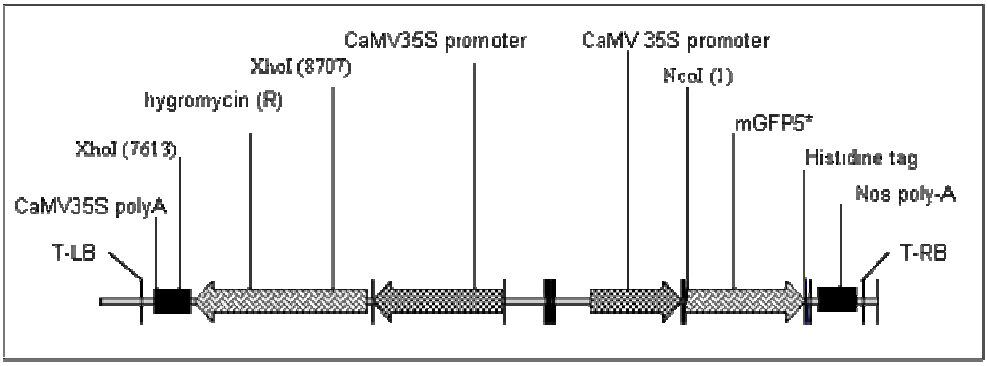

Fig. (5): Schematic representation of the T-DNA of the pCAMBIA 1302 plasmid used for transformation of two high oleic *H. annuus* L. genotypes (cv.capella and SWSR2 inbred line) containing the *mgfp5* and *hpt* genes under different constitutive promoters.

8.1- *Agrobacterium* culture and transformation method

Selection of *Agrobacterium* strains LBA4404 and culturing were done on YEB medium plates as previously described in (II.7.3.1) with addition of the appropriate antibiotic (Table 8). 100 shoot apices of cv.capella were transformed using the previous established *Agrobacterium* infiltration protocol (II.7.5) whereas 80 shoot apices of SWSR2 inbred line were transformed using the previous established particle bombardment protocol (II.7.5) using *mgfp5* gene.

9- Analysis of transformants

- ***Buffers and solutions***

Bradford solutio

70 mg coomassie blue G in 50 ml ethanol.

100 ml 85% (v/v) phosphoric acid.

fill up to 200 ml with distilled water.

Bradford working solution

1- Bradford stock solution diluted 1:5 with distilled water.

2- Overnight at 4°C.

3- Filtration.

CTAB buffer

100 mM Tris-HCl pH 8.0

20 mM EDTA pH 8.0

1.4 M NaCl

3.5 % (w/v) CTAB

0.2 % (v/v) β-mercaptoethanol

DNA extraction buffer (modified after Dellaporta et al., 1983)

100 mM Tris-HCl pH 8.0

50 mM EDTA pH 8.0

500 mM NaCl

10 mM β-mercaptoethanol

RNase-A

10 mM potassium acetate

10 % (v/v) 1 M Tris-HCl pH 7.40

10 mg RNAse-A

10X TAE buffer

0.4 M Tris

0.2 M Na-acetate

0.01 MEDTA

Tris-EDTA (TE buffer) pH 8.0

10mM Tris-HCl

1 mM EDTA pH 8.0

20X SSC

3 M NaCl

0.3 M Na-citrate

Denaturing solution

0.5 M NaOH

1.5 M NaCl

Neutralizing solution

1 M Tris-HCl pH 8.0

1.5 M NaCl

100X Denhard ´s solution

2 % (w/v) PVP

2 % (w/v) BSA

2 % (w/v) Ficoll (400)

Hybridization solution

0.6 M NaCl

0.1 M Pipes pH 6.8

1.0 mM EDTA pH 8.5

0.1 % (w/v) SDS

10X Denhard ´s solution

10 µg/ml sperm DNA

Washing solution

2X SSC

0.1 % (w/v) SDS

DNA loading buffer

25 mg bromophenol blue

25 mg xylencyanol

1 ml 10X TAE buffer

3.45 ml 87 % (v/v) glycerol

5.55 ml sterile distilled water.

9.1- Histochemical GUS assay

Beta-glucuronidase (GUS) activity was assayed according to Jefferson *et al*., (1987). Regenerated shoots (five weeks old in all transformation experiments testing different parameters, 8 weeks old in each final representative transformation experiment and 12-14 weeks old in stable transformation experiment) were immersed in Gus staining solution, (0.1M Na_2HPO_4; PH 7, 10 mM NaEDTA, 0.5 mM K-ferricyanid, 0.5 mM K-ferrocyanid, 0.1 % (v/v) Triton-X-100, 1 mM X-Gluc (5 bromo-4-chloro-3-indolyl glucuronide) and 20 % (v/v) methanol) (Kusogi *et al*.,1990) and vacuum infiltration was applied at 200 mbar for 10 min, then incubated overnight in dark at 37 °C. Before microscopic analysis, chlorophyll was bleached by extraction in ethanol series (70%, 96%) for 24 h. Untreated explants were cultured under identical conditions and served as negative control. By this assay qualitative data concerning the specificity of the *gus* gene expression in tissue were obtained. Transient as well as stable expression could be studied. After the histochemical assay the positive plants were kept for PCR analysis.

9.2- Fluorometric GUS assay

The fluorometric GUS assay was performed according to Jefferson *et al*., (1987). For all the transformation experiments, testing different parameters, the fluorometric GUS assay was performed four weeks after co-cultivation except for the bacterial strains experiment, fluorometric GUS activity was measured after two and four weeks. In each final representative transformation experiment, using the optimized protocol, the fluorometric GUS assay was performed 8 weeks after co-cultivation. In stable transformation experiments fluorometric GUS activity was measured 5, 10 and 12-14 weeks after co-cultivation. Plant tissue from transformed and non-transformed samples was ground with a pestle and mortar in the presence of liquid nitrogen. Tissue was homogenized in microcentrifuge tube with extraction buffer (2 ml/g ground tissue); (50 mM NaH_2PO_4; pH7, 10 mM EDTA; pH 8, 0.2 % (v/v) Triton X-100 and 10 mM ß-mercaptoethanol), centrifuged to pellet debris and the supernatant collected. Crude protein content of the extract was quantified according to Bradford, (1976), mixed with 500 µl MUG solution: (1 mM 4-methylumbelliferyl- ß -glucoronide in 20% (v/v) methanol). The reaction carried out in the dark at 37 °C for 1 h. and stopped

with 400 µl 0.2 M Na_2CO_3. Preparation was analyzed in Fluoro-Max spectro fluorometer, the fluorescence was recorded at an excitation wavelength of 365 nm and an emission of 455 nm. The readings were compared to readings of 4-MU standards of varying concentrations and were plotted against time to determine the amount of MU produced. GUS activity was calculated as micromoles of 4-MU formed /mg protein /min.

9.2.1- Test of GUS linearity

Pre-transformation experiment was conducted using *Agrobacterium* strain GV3101 as it was routinely used in our lab. In this experiment the fluorometric GUS assay was performed as previously described in (II.9.2) and the fluorescence was recorded for three replicates after 15, 30, 60, 90 and 120 min in order to determine the time at which the Gus activity is linear.

9.2.2- Effect of methanol on endogenous β- glucuronidase activity

In the first transformation experiment which tests the effect of different *Agrobacterium* strains, fluorometric GUS assay was performed as mentioned before with and without addition of 20% (v/v) methanol to the MUG solution. This experiment was carried out to study the effect of methanol on the endogenous β-glucuronidase activity as well as the transformation frequency of various tested bacterial strains.

9.3- Histological GFP assay

In the present investigation, GFP was visualized using a fluorescence microscope with a 100 W-Quecksilver lamp, a BP 450-490 nm excitation filter and 505 nm barrier filter.

9.4-Fluorometric GFP assay

Fluorometric GFP assay was performed according to Müller *et al*., (2001). Plant tissue was ground with a pestle and mortar in two volume of extraction buffer (50 mM Tris-HCl; pH 8.0, 10 mM EDTA, 10 mM dithiotreitol, 18 % (v/v) glycerol) in the presence of liquid nitrogen. The extraction was centrifuged to pellet debris and the supernatant was collected. Protein content of the extracts was determined by method

of Bradford, (1976). Preparations were diluted with buffer to 75 µg/ml and analyzed in the Fluoro-Max spectro fluorometer using the excitation filter at 490 nm combined with the emission filter at 525 nm.

9.5- DNA isolation and molecular analysis of transgenic plants

- ***Genomic DNA isolation***

For genomic DNA extraction from plant material, different protocols were tested.

❖ *DNA extraction (modified after Dellaporta et al., 1983)*

About 0.1 g fresh young leaves were collected directly in 1.5 ml eppendorf tube and shock-frozen in liquid nitrogen. After homogenisation with micropestle 20 µl extraction buffer (II.9) + 2 µl 20 % (w/v) SDS solution were added, incubated at 65°C for 5 min. after which 10 µl 5 M potassium acetate solution was added and incubated for 2 min at 4 °C. Centrifugation was carried out for 5 min. at 14,000 rpm and 4 °C. The supernatant was transferred to a new eppendorf tube, 17.5 µl isopropanol was added and incubation was at room temperature for 5 min. The DNA pellet was collected by centrifugation at 14,000 rpm for 5 min. at 4 °C. Washing was performed 2 times with 70 % (v/v) ethanol. Finally the pellet was dried at RT and then re-suspended in TE buffer.

❖ ***DNeasy plant Mini Kit***

100 mg of fresh young leaves were taken and the DNA was extracted according to the manufacturer instructions

❖ ***DNA extraction using CTAB method (Doyle and Doyle, 1987; Cullings, 1992)***

Plant material (0.3-0.9 g) was homogenised in liquid nitrogen with pestle and mortar and transferred into 50 ml centrifuge tubes. The plant powder was vigorously mixed with 7.5 ml CTAB extraction buffer (pr-heated at 60°C) and incubated at 60 °C for 30 min. The mixture was then allowed to cool at RT for 5 min. To each sample 1 volume of Chlorophorm: isoamylalcohol (24:1) was added and solutions mixed by gentle inversion. After 5 min. the samples were centrifuged at 14,000 rpm for 15 min. at RT and the supernatant was transferred to a new tube. For DNA precipitation, 2/3

volume of pre-cooled Isopropanol was added to the pure supernatant and incubated for 30 min. at RT. Samples were centrifuged at 14,000 rpm, 4 °C for 5 min., the supernatant was removed and 10 ml of 76 % (v/v) ethanol + 10 mM NH_4 acetate was added and the mixture left at RT for 20 min. The DNA pellet was collected by centrifugation at 14,000 rpm, 4 °C for 10 min., allowed to dry at RT for 10 min. and then 1 ml of 10 mM Tris-HCl pH 8.00 plus 10 µl RNase solution (10 mg/ml) were added. After 30 min. incubation at 37°C, DNA was precipitated by the addition of 2 V 10 mM Tris-HCl pH 8.00, 0.3 V of 7.5 M NH_4 acetate and 2.5 V of absolute ethanol and incubated at -20 °C overnight. The samples were centrifuged at 14,000 rpm for 20 min. at 4 °C, the supernatant was removed and the DNA pellet was then washed 2 times with 70 % (v/v) ethanol and allowed to dry at RT for 10-15 min. DNA pellet was finally dissolved in 40-100 µl 10 mM Tris-HCl ph 8.00. DNA concentration was quantified by spectrophotometer at 260 nm. An OD of 1.0 at 260 nm equals dsDNA concentration of 50 µg/ µl (Sambrook *et al.*, 1989). DNA concentration was calculated in µg/ µl according to the following formula:

$$\text{DNA concentration} = (Abs._{260} \times \text{dilution} \times 50) /1000$$

The quality of DNA was determined by gel electrophoresis (0.8 % (w/v) agarose).

- ***DNA restriction***

Restriction endonuclease digestion was carried out under conditions recommended by the manufacturer. About 50-100 ng of genomic DNA isolated from both untreated and transformed plants (from the final representative experiments after 14-16 weeks development and from T_0 and T_1) were digested with 2 U *Eco*RI in 10 µl reaction:

50-100	ng DNA
0.2 µl	*Eco*RI (1 U/µl)
1 µl	Buffer 10X

complete to 10 µl with distilled water

The digestion proceeded for 2 hours at 37 °C in 10 µl reaction.

9.5.1- Polymerase chain reaction

DNA from transformed, positive GUS assay, and random non transformed plants (from the final representative experiments after 14-16 weeks development and from T_0 and T_1) was extracted according to the CTAB method (II.9.5). The PCR (Mullis and Faloona, 1987) specifically amplifies DNA sequences between defined synthetic primers, designed according to the target DNA sequence. Detection of the *gus*, *mgfp5 and nptII* genes in the samples was conducted using PCR with the following primers: *gus* primer 5`-ATG TTA CGT CCT GTA GAA AC-3´ and 5´-CTT CAC TGC CAC TGA CCG GA-3´, which were designed to amplify approximately 830-bp DNA fragment of the *gus* cDNA, *nptII* primer 5´-ACA AGA TGG ATT GCA AGG-3´and 5´-AAC TCG TCA AGA CGA TAG-3´ which amplify approximately 804-bp DNA fragment and *mgfp5* primer 5´-AAA GGA GAA GAA CTT TTC ACT-3´ and 5´- TTT GTA TAG TTC ATC CAT GCC- 3´-, which amplify approximately 800-bp fragment. To test the possibility of bacterial contamination of the plant tissue, the following primer was used: *virA* primer 5´-TCT ACG GTC ATG GTC CAC TAG ACG-3´ and 5´-TGC TGC TCA ACT GCT ACG CCA GCT-3´ to amplify a 500 -bp fragment of the *Agrobacterium* chromosomal *vir*A (Bond and Roose, 1998).

PCR reaction was performed in 50 µl reaction mix containing:

5 µl	1.5 mM $MgCl_2$
5 µl	10X Taq DNA polymerase buffer
0.75 µl	0.2 mM dNTP
12.5 µl	0.25 µM of each primer
50-100 ng	*Eco*RI digested genomic DNA
2 U/50 µl	Taq DNA polymerase

As a positive control, the corresponding plasmid or *Agrobacterium* DNA was used as a template (*Agrobacterium* DNA was isolated according to Maniatis *et al*., 1982). DNA samples were denatured for 5 min. at 95°C and amplified during 32 cycles, denaturation for 1 min. at 95°C, annealing for 1 min. at 64°C for *gus*, *mgfp5*, *pica*, *virA* and at 58°C for *nptII*, extension for 1 min. at 72°C. Cycling was closed with a final extension step for 10 min. at 72°C. Amplified products were mixed with 6X loading dye, electrophoresed using the horizontal electrophoresis apparatus on a 8%

(w/v) agarose gel electrophoresis was carried out in 1X TAE buffer for 1h at 70 mA. The gel was stained in TAE buffer containing 40 µl/l of 0.1% (w/v) ethidium-bromide and the DNA was visualized with the ethidium-bromide on a UV transilluminator (Sambrook *et al*., 1989) at 305 nm.

9.5.2- Southern blot analysis

a- Preparation of DNA probe

❖ ***Mini plasmid isolation from E.coli***

Mini preparation of plasmid DNA from *E. coli* DH5α carrying either the plasmid pBI121 or pCAMBIA1302 was performed as previously described in section (II.7.3.4)

❖ ***DNA extraction from agarose gel***

PCR reaction was carried out as previously described (II.9.5.1) using 50-100 ng of DNA, isolated from *E. coli* carrying the pBI121, or pCAMBIA1302 plasmid as a template. DNA sample was run on 0.8% (w/v) agarose gel and examined under UV light. The DNA fragment of interest was sliced out from agarose gel and treated according to gel extraction Kit following the manufacturer instructions.

b- Southern hybridization

- ***DNA restriction and gel electrophoresis***

Restriction endonuclease digestion was carried out under conditions recommended by the manufacturer. 20 µg of genomic DNA were digested with *Eco*RI. The digestion was performed for some representative T_0 plants and T_1 plantlets of stable transformation experiments and some representative T_0 plants from *mgfp5* transformation experiments for 3 h at 37°C with 40 U of restriction enzyme in 50 µl reaction:

20 µg	genomic DNA
4 µl	*Eco*RI or *Hind*III (10U/µl)
5 µl	Buffer 10X

complete to 50 µl with distilled water

Electrophoresis was carried out as previously described for 3h at 70 mA.

- ***Transfer of restriction fragments to membrane***

The agarose gel containing the separated DNA fragments was treated for 15 min. in 0.25 M HCl at RT, then shaked for 30 min. in denaturing solution and for another 30 min. in neutralizing solution. The DNA fragments were transferred from the gel to the Hybond-N^+ nitrocellulose membrane overnight with 20 X SSC buffer. For fixation of the DNA fragments, the membrane was exposed to UV light for 5 min. and backed at 80°C for 2 h.

- ***Labeling of DNA***

❖ ***Labeling of DNA using ECL™ Kit***

DNA probe was labeled using ECL™ Kit according to the manufacturer instructions, in addition, the sensitivity of the method was tested by dot blot experiment. The pBI121 plasmid was isolated as mentioned in (II.7.3.4) and different concentrations (10, 20, 40, 80, 160, 320, 640 and 1100 ng) were used for dot blot.

❖ ***Labeling of DNA using [α-^{32} P]***

The DNA probe was labeled with [α-^{32} P]-dCTP using the "Random primed hexalabeling DNA Kit" and 50-100 ng DNA were used as a template. 15 µl probe in 28.5µl sterile distilled water and 10 µl hexanucleotide in a total volume 40 µl was incubated for 5 min. at 95°C for denaturation, then quick chilled in ice. Finally, 3 µl Mix C, 2 µl [α-^{32} P] –dCTP and 1 µl Klenow enzyme (5 U/µl), were added and incubated for 10 min. at 37°C. After incubation, 4 µl dNTPs was added and re-incubated for 5 min. at 37°C. The reaction was stopped with 50 µl TE buffer; pH 8 and the probe were allowed to pass through a sephadex column (to clean the probe). Before using the probe, it was incubated at 95°C for 5 min. for denaturation and quick chilled on ice.

- ***Hybridization process***

The pre-hybridization was carried out by using 50-100 ml hybridization solution at 65°C for at least 3 h without labeled DNA. The hybridization solution was refreshed and the hybridization process performed overnight at 65°C with labeled DNA probe. The membrane was washed 3 times with 50 ml washing buffer for 20 min. each at 65°C. The membrane was then exposed to kodak hyper-film for 3-5 days at -70°C.

RESULTS

In vitro culture system

The main aim of these experiments was to establish an efficient and reproducible regeneration protocol suitable for transformation experiments.

1- Germination efficiency of different *H. annuus* L. genotypes

Data in Table 9 showed the differences in germination efficiency of different tested *H. annuus* L hybrids (cv.capella, Prolic-204 and Orbaril) and inbred lines (SWSR1 and SWSR2). SWSR2 inbred line and cv.capella achieved the highest germination percentage (98.3 and 97.7 %), respectively, followed by SWSR1 inbred line and Prolic-204 whereas, Orbaril showed a germination percentage of 89.7%.

Table (9): Germination efficiency of different tested genotypes.

Genotype	Germination (%)
cv.capella	97.7
Prolic-204	92.3
Orbaril	89.7
SWSR1	96.3
SWSR2	98.3

Number of seeds = 300

2- Shoot induction

In these investigations screening of different *H. annuus* L. hybrids (cv.capella, Prolic-204, Orbaril) and inbred lines (SWSR1and SWSR2) were performed using two shoot induction media (SIM1 and SIM2) either with intact or split shoot apices (Fig. 6, 7, 8, 13A and Table 10, 11). Two simple but efficient shoot induction media combination were selected for these experiments. Data in Fig. 6 revealed that shoot induction was possible for all tested *H. annuus* L. genotypes on both tested shoot induction media (SIM) using intact or split shoot apices. Culturing of intact shoot apices on SIM1 achieved the highest shoot induction frequency (45%) from cv.capella and the lowest frequency (6%) from Orbaril, while SWSR2 inbred line and cv.capella gave the highest shoot induction frequency (64 and 50%), respectively, using the same

type of explant on SIM2 (Fig. 6 A and B). These results were confirmed with the data of regeneration efficiency (defined as scores) which generally showed that using SIM2 enhanced the regeneration efficiency in all tested genotypes comparing with SIM1. The mean regeneration efficiency of regenerated shoot of cv.capella and SWSR2 on SIM1 using intact shoot apices was 2.3 and 2.2 regeneration score, respectively, while it increased to 3.8 and 4.0, respectively, on SIM2 using the same type of explant (Table 10).

It was clear that using split shoot apices improved shoot induction frequency as well as the regeneration efficiency independently of genotype and shoot induction media comparing with intact shoot apices (Fig. 6 C, D and Table 10). Shoot bud differentiation took place in the explants of all tested genotypes on the frontal portion of the bisected shoot apices. The potential for direct shoot induction was restricted only to the meristem adjacent tissue and absent in the opposite extreme of the explant (Fig. 13A). High shoot induction frequency (60 and 56%) was observed for both selected media (SIM1 and SIM2), respectively, with cv.capella using split shoot apices (Fig 6C and D). Most interesting was that the combination of split shoot apices of SWSR2 with SIM2 increased the shoot induction frequency 3.5 fold compared to SIM1, while the increase in Orbaril using the same combination was 2.5 fold (Fig 6 C and D). Moreover, using split shoot apices with SIM2 increased the regeneration efficiency in all tested genotypes. The regeneration efficiency of regenerated shoot from cv.capella using split shoot apices and SIM2 increased 2.3 fold than using the same explant with SIM1 (Table 10). Regenerated shoots of cv.capella showed a superior vigor in relation to the shoots from other genotypes (Fig. 7). Analysis of the results (Fig. 6B, D and Table 10, 11) revealed that the interaction between genotype and media was more pronounced for the given experiments.

With regard to the number of regenerated shoots per explant, generally, using intact shoot apices with both shoot induction media induced only one shoot per explant on the other hand, using split shoot apices encouraged multi shoot induction per explant in all tested genotypes. The most commonly observations that using SIM1 induced one or two shoots per explant, while using SIM 2 induced up to four shoots per bisected explant without influence on the regeneration efficiency (Fig. 6E, F, 13A and Table 10). In SWSR2 57.8% of regenerated explants have one shoot and 42.2%

have two shoots using SIM1, whereas, 14.3% of regenerated explants have one shoot, 17.4% have two shoots, 36.8% have three shoots and 31.5% have four shoots using SIM2.

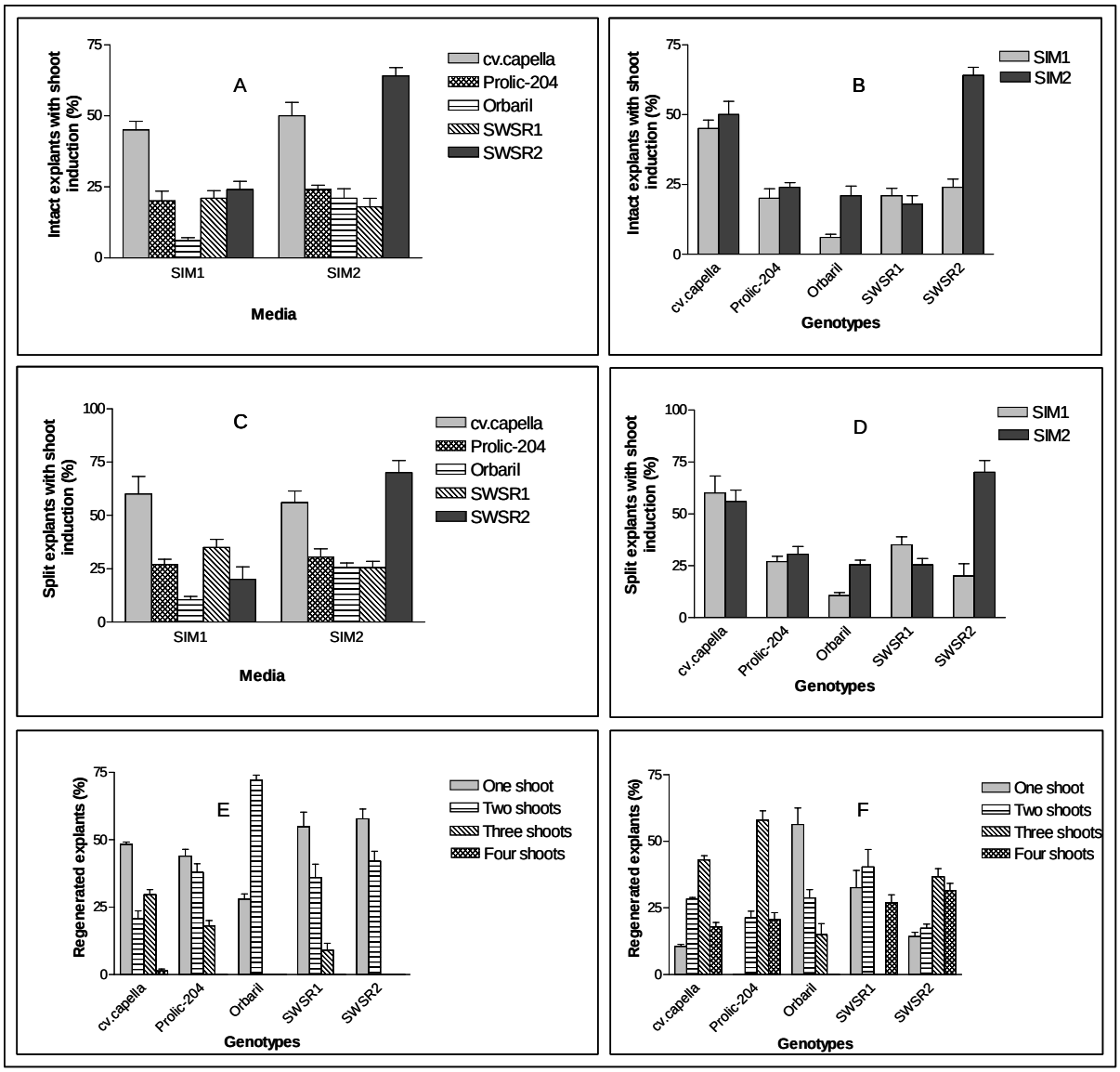

Fig. (6): Direct organogenesis of intact and split shoot apices from different *H. annuus* L. genotypes and the variability of shoot numbers per regenerated explant on different shoot induction media (A) regeneration frequency of intact shoot apices (percentage of intact explants with shoot induction) in the tested media, (B) response of the genotypes to the shoot induction media, (C) regeneration frequency of split explants (percentage of split shoot apices with shoot induction) in the tested media, (D) response of the genotypes to the shoot induction media, (E) percentage of variable numbers of regenerated shoots of various tested genotypes on SIM1, (F) percentage of variable numbers of regenerated shoots of various tested genotypes on SIM2. Data are means of 4 replicates ± SE.

Table (10): Regeneration efficiency of split and intact shoot apices of different tested genotypes on various shoot induction media.

Genotype	Regeneration efficiency *			
	SIM1		SIM2	
	Intact shoot apices	Split shoot apices	Intact shoot apices	Split shoot apices
cv.capella	2.3 ± 0.17	2.1 ± 0.24	3.8 ± 0.35	4.8 ± 0.12
Prolic-204	1.3 ± 0.24	1.0 ± 0.17	2.4 ± 0.34	2.3 ± 0.29
Orbaril	1.7 ± 0.26	1.5 ± 0.24	2.4 ± 0.37	1.8 ± 0.35
SWSR1	1.4 ± 0.11	1.4 ± 0.2	2.6 ± 0.34	2.1 ± 0.18
SWSR2	2.2 ± 0.23	2.6 ± 0.40	4.0 ± 0.48	4.2 ± 0.31

* Regeneration efficiency was expressed as a score (1 – 5), negative result = 1, below average = 2, average = 3, above average = 4 and excellent =5. Values are means of 4 replicates ± SE.

An expressive difference between media SIM1 and SIM2 was the vigour of the induced shoots. Induced shoots on SIM2 were normal with well formed green leaves, in contrary to those from SIM1 appearing weak and pale leaves with white margins (Fig. 7).

In addition, data in Table 11 revealed that shoot differentiation was appeared in the meristem adjacent tissue of cv.capella and Prolic-204 after 14 days and 21 days for both inbred lines whereas, the explants extended at least 2 times. The highest callus formation percentage was 35% from SWSR1 inbred line, while cv.capella achieved the lowest callus formation percentage (15%) among the tested genotypes.

Hyperhydratation is known as a very common problem in sunflower regeneration experiments. However, the results showed a low (5-7%) incidence of hyperhydrated explants or regenerated shoots (Table 11).

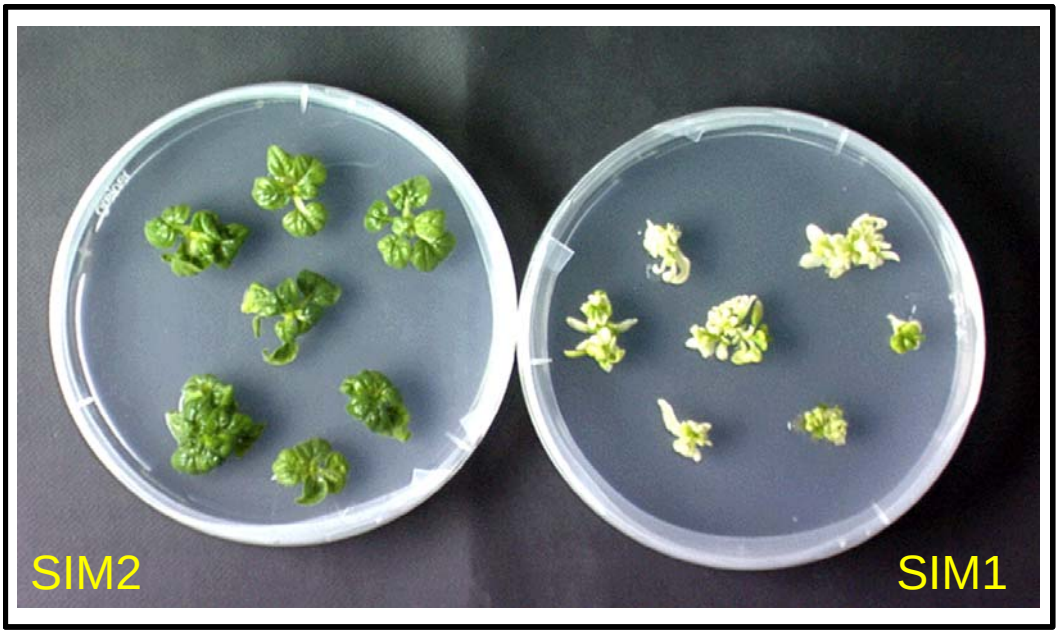

Fig. (7): Effect of shoot induction media (SIM1 and SIM2) on direct regeneration of split shoot apices of *H. annuus* L. tested genotypes, e.g. cv.capella.

Table (11): General performance of different tested genotypes, in relation to plant regeneration via direct organogenesis.

Parameters	cv.capella	Prolic-204	Orbaril	SWSR1	SWSR2
Shoot primordial initiation (days)	14	14	17	21	21
Mean of explants with shoot induction (%)	58	28.5	18	30.25	45
Callus formation (%)	15	16	17	35	30
Shoots with Hyperhydratation (%)	5	7	10	10	7
Number of shoots per explant	1-3	1-4	1-3	1-4	1-4

In conclusion, Fig. 8 illustrated a summary of all experiments carried out for screening different *H. annuus* L. genotypes as well as comparison between intact and split shoot apices using two different shoot induction media (SIM1 and SIM2).

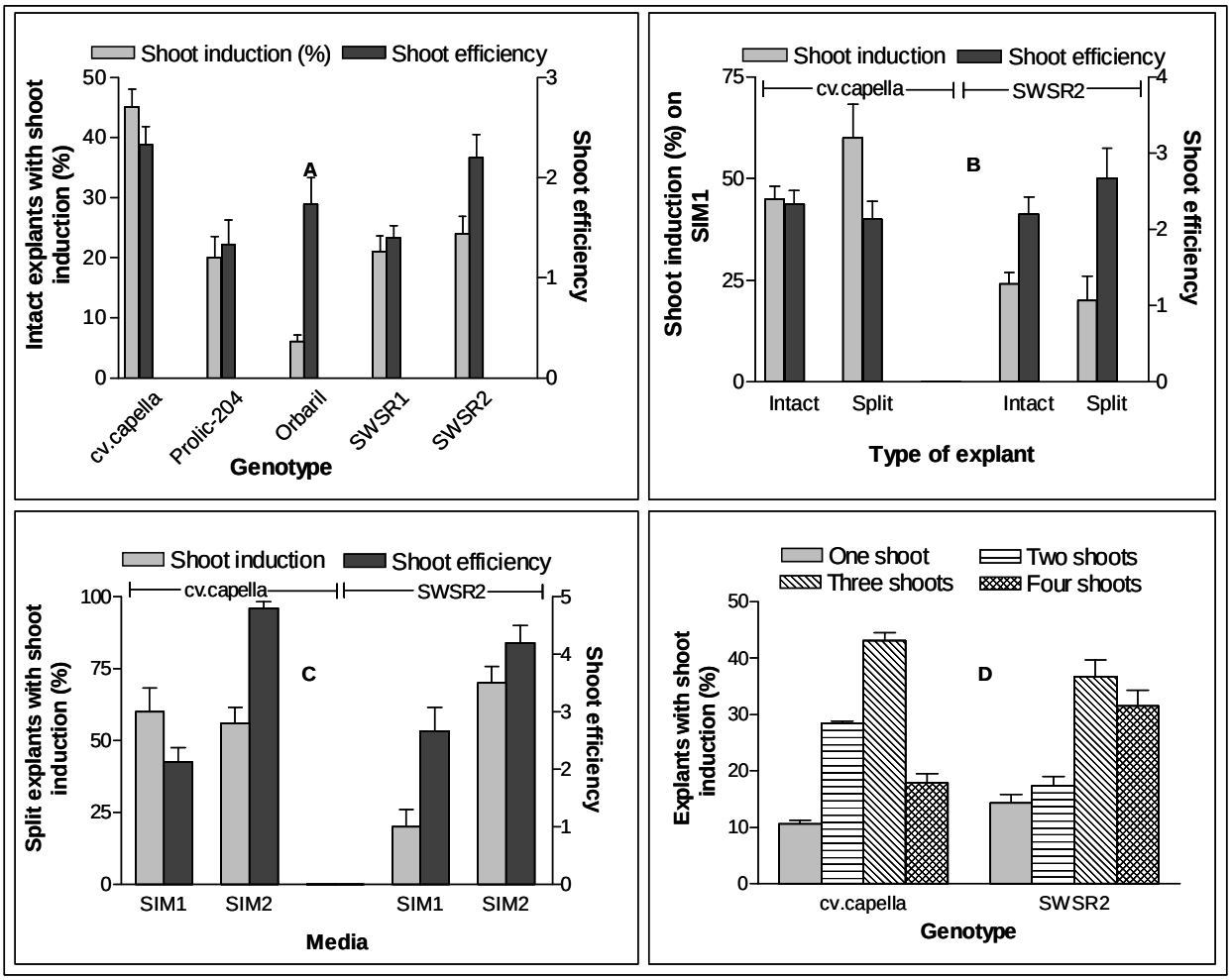

Fig. (8): Summary of regeneration protocol of various *H. annuus* L. genotypes. A: test of different genotypes on SIM1 using intact shoot apices, B: test of different type of explants from high oleic genotypes, cv.capella and SWSR2 inbred line on SIM1, C: test of different regeneration media with split shoot apices from cv.capella and SWSR2 inbred line and D: number of shoots per regenerated explants on SIM2 using split shoot apices from cv.capella and SWSR2 inbred line.

These results were summarized in the following points:

- Cv.capella and SWSR2 were chosen among different tested genotypes for subsequent experiments because of the high frequency of shoot induction (45 and 24%) and efficiency (2.3 and 2.2 regeneration score), respectively on SIM1 (Fig. 8A).
- Using split shoot apices with SIM1 enhanced the shoot induction frequency of cv.capella 15% and shoot efficiency of SWSR2 1.2 fold (Fig. 8B).

- SIM2 with split shoot apices achieved high regeneration frequency (56 and 70%) and the highest regeneration efficiency (4.8 and 4.2) of cv.capella and SWSR2, respectively (Fig. 8C and D).
- Despite of the multi shoot induction, the most efficient shoots were obtained from cv.capella and SWSR2 on SIM2.

3- Shoot elongation

In an attempt to elongate the regenerated shoots of cv.capella and SWSR2 inbred line on SIM2, two shoot elongation media (SEM1 and SEM2) were used.

Analysis of the results in Table 12 and Fig. 9 showed that no elongation response was obtained in regenerated shoots of both genotypes using SEM1, while using SEM2 supplemented with different concentration of gibberellic acid showed different results. No elongation appeared when using SEM2 containing 0.29 µM GA_3 of both genotypes. The highest shoot elongation response was 2-3 cm on SEM2 supplemented with 1.45 µM GA_3 in both genotypes regardless of the length of started regenerated shoots. The most commonly observation was that using SEM2 containing the highest GA_3 concentration encouraged floral bud and callus formation on the elongated shoots in both genotypes (Fig. 9).

Table (12): Elongation response of cv.capella and SWSR2 inbred line on different tested elongation media.

Genotype	SEM1	SEM2		
		0.29 µM GA_3	0.58 µM GA_3	1.45 µM GA_3
cv.capella	+	+	++	+++[A]
SWSR2	+	+	++	+++[A]

No elongation = +, 1-2 cm shoot length = ++, 2-3 cm shoot length = +++, 3-4 cm shoot length = ++++, > 4 cm shoot length = +++++. This scoring system is regardless the length of the started regenerated shoots. (A) Elongated shoots formed floral buds and calli at the lower part.

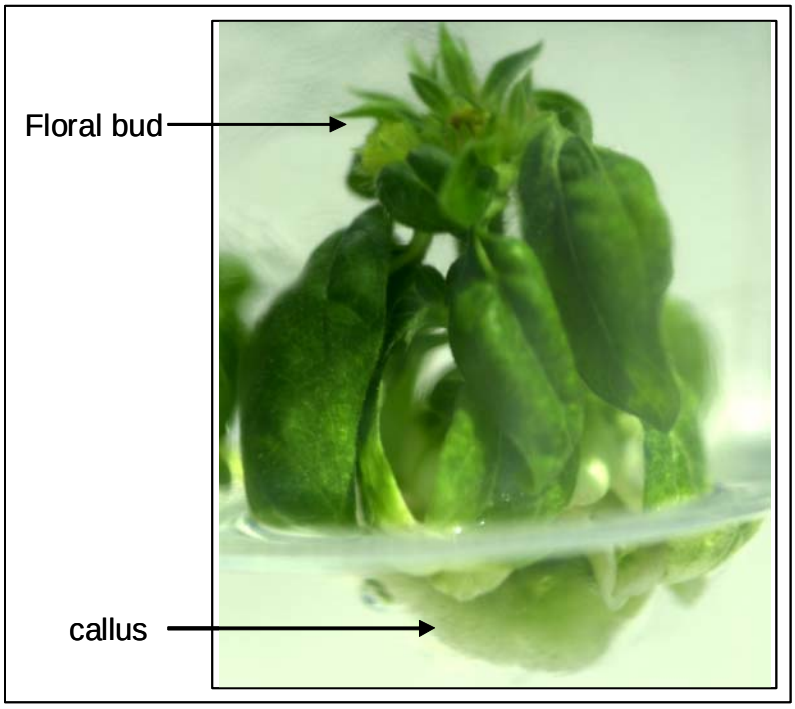

Fig. (9): Elongated shoots of tested genotypes on SEM2 supplemented with 1.45 µM GA3, e.g. SWSR2.

4- Root induction

The well developed shoots of cv.capella and SWSR2 inbred line from both groups (elongated shoots on SEM2 and non-elongated shoots) were cultured on two different root induction media (RIM1 and RIM2). Data of rooting were presented in Fig 10, 11, 13B and Table 13, 14.

Results in Fig 10 revealed that root induction of non elongated shoots was efficient on both root induction media (60-90%) with respect to genotypes. The highest root induction frequency of cv.capella was 90% on RIM2 whereas, in SWSR2 inbred line was 66% on RIM1 (Fig. 10A) and without formation of floral buds. No rooting was achieved from culturing of elongated shoots on both induction media, in addition to inflorescence formation on these shoots (Table 13). The interaction between genotypes and root induction media was obvious in Fig. 10B.

Both media were efficient for root induction, but no shoot elongation was promoted under these growth conditions.

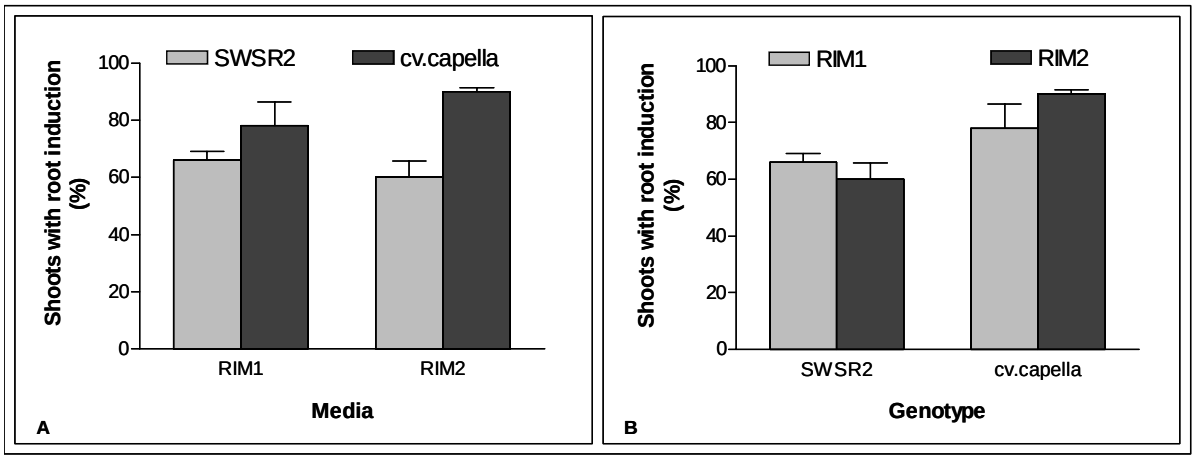

Fig. (10): Root induction of cv.capella and SWSR2 inbred line on different root induction media (A) percentage of shoot with root induction in the tested media and (B) response of the genotypes to the root induction media. Data are means of three replicates ± SE.

Table (13): Number of roots per regenerated shoot and root description of cv.capella and SWSR2 inbred line on various root induction media.

Genotype	RIM1				RIM2			
	A		B		A		B	
	No. of roots / reg. shoot	Root description	No. of roots / reg. shoot	Root description	No. of roots / reg. shoot	Root description	No. of roots / reg. shoot	Root description
cv.capella	0.0*	-	10.67 ± 0.33	Short, thick	0.0*	-	4.0 ± 0.58	Long, thin
SWSR2	0.0*	-	9.0 ± 1.16	Short, thick	0.0*	-	3.33 ± 0.88	Long, thin

A= elongated shoots and B= non-elongated shoots. (*) Elongated shoots showed early flowering and reg. = regenerated. Values are means of 3 replicates ± SE.

In order to evaluate the number of roots per regenerated shoots and root description, abundant and vigorous root primordia were induced within 4-5 days

(Table 14), with a simple RIM1 medium containing lower sucrose concentration, vitamins and growth regulators and without $AgNO_3$ in relation to RIM2 medium. Results in Table 13 and Fig 11 showed that the mean number of roots per regenerated shoots of cv.capella was 10.6 on RIM1 whereas, it was 4.0 on RIM2. Using RIM1 increased the root density in SWSR2 from 3.3 on RIM2 to 9.0.

It was obvious in both genotypes that the induced roots on RIM1 were short, thick and efficient while, those on RIM2 were long and thin (Table 13 and Fig. 11).

Therefore, RIM1 was selected for all subsequent regeneration experiments.

Table (14): Root primordial initiation and root induction of cv.capella and SWSR2 inbred line on root induction media.

Genotype	cv.capella	SWSR2
Root primordial initiation (days)	5	4
Mean of shoots with root induction (%)	84	63

Fig. (11): Effect of different root induction media (RIM1 and RIM2) on root formation and root efficiency of *H. annuus* L. tested genotypes, e.g. SWSR2 inbred line.

5- Shoot elongation and root system development

In an effort to elongate the plantlets of cv.capella and SWSR2 inbred line, the regenerated shoots on SIM2 and directly rooted on RIM1 were transferred to shoot elongation and root development media (SER1 and SER2). Table 15, 16 and Fig. 12, 13C illustrated that elongation was possible for both genotypes on both media.

Shoot elongation was induced to a high extent (more than 4 cm regardless the length of the regenerated shoots) by using simple MS medium contains vitamins free of growth regulator (SER2), in addition to development the rooting system and its efficiency (Fig. 12). Using SER2 decreased the frequently encountered *in vitro* flowering problem to 5% and 7% in SWSR2 and cv.capella, respectively. Shoot elongation incidence was observed within a period of 21 days for both genotypes (Table 16).

Despite of the efficiency of shoot elongation on SER1 medium in the presence of GA_3, the shoots became thin and slender with small leaves and developed the problem of premature flowering in both genotypes. Consequently, SER2 medium was chosen to elongate the plantlets of cv.capella and SWSR2 inbred line.

Table (15): Elongation response and development of rooting system of cv.capella and SWSR2 inbred line on different shoot elongation and root development media.

Genotype	SER1	SER2
cv.capella	++++ (a)	+++++ (b)
SWSR2	+++ (a)	+++++ (b)

No elongation = +, 1-2 cm shoot length = ++, 2-3 cm shoot length = +++, 3-4 cm shoot length = ++++, > 4 cm shoot length = +++++. This scoring system is regardless the length of the started regenerated shoots. (a) the shoots became thin and slender and developed the problem of premature flowering (b) Developed rooting system.

Fig. (12): Effect of shoot elongation and root development media (SER1 and SER2) on shoot elongation and development of rooting system of *H. annuus* genotypes, e.g. cv.capella.

Table (16): General performance of cv.capella and SWSR2 inbred line, in relation to plantlets elongation, acclimatization and seed production.

Parameters	cv.capella	SWSR2
Shoot elongation (days)	21	21
In vitro flowering (%)	7	5
Plantlets surviving by soil transfer (%)	80	85
Time to flowering (weeks)	12	13
Number of seeds/head	1-9	1-11
Plant height (cm)	35	50

6- Plant acclimatization

In the final step of this established protocol, normally elongated plantlets of both genotypes were transplanted *ex vitro* in small pots in garden soil to growth chambers (Fig. 13D). The frequency of survival plantlets after transferring to soil was high (over 80%) and many plants were grown to maturity, flowering (after 12-13 weeks) and seed production (9-11 seeds/head)(Table 16 and Fig. 13E, F). Phenotypically some plants were weak, sensitive, branched with flowers, and the main axis was frequently shorter (about 35 cm in cv.capella and 50 cm in SWSR2 inbred line) than the side shoots

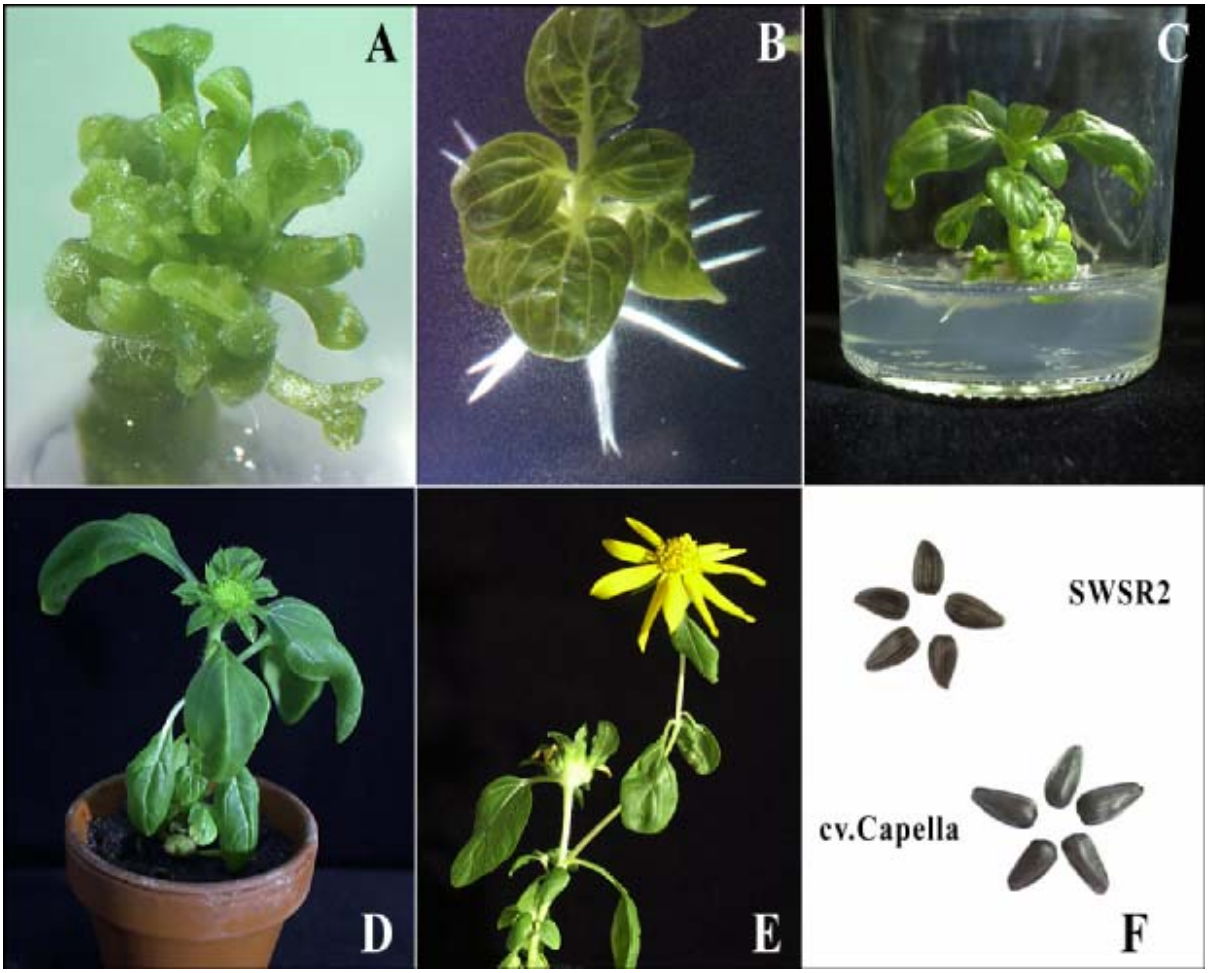

Fig. (13): Direct regeneration of cv. capella and SWSR2 inbred line: (A) multiple shoot at the meristem adjacent tissue after 3 weeks on shoot induction medium SIM2, (B) regenerated shoots in root induction medium RIM1, (C) plantlet in shoot elongation and root development medium SER2, (D) elongated plantlet transferred to a pot in *ex vitro* culture, (E) flowering regenerated plant under growth chamber conditions and (F) viable seeds of the regenerated plants.

In conclusion, regeneration of split shoot apices of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line on SIM2 achieved multi shoot induction in addition to the highest shoot induction frequency in both genotypes. Culturing of regenerated shoots directly on RIM1 increased the root induction frequency and efficiency with respect to genotypes and overcame the high frequency incidence of *in vitro* flowering. Elongation of the regenerated shoots with initial roots on SER2 medium increased shoot length and developed the rooting system. Finally, acclimatization of regenerated plantlets showed further development reaching the flowering stage and seed production.

The previous established regeneration protocol has been repeated three times and the obtained results were similar. This proved the efficiency and reproducibility of this protocol. Therefore, it was used in all subsequent transformation experiments.

Transformation of shoot apices using *gus* reporter gene.

1- *Agrobacterium* elimination

Prior to transformation experiments cefotaxime and carbenicillin, which are widely used antibiotics for *Agrobacterium* elimination after co-cultivation period in transformation experiments, were tested for their effect on shoot induction frequency and efficiency using different concentrations. The non transformed split shoot apices were placed on SIM2 supplemented with different antibiotics concentrations and the data were presented in Fig. 14, 15 and Table 17, 18.

- ***Effect of cefotaxime on plant regeneration***

Fig. 14 and Table 17 showed that no obvious differences were detected in shoot induction frequency (regeneration frequency) and efficiency between 125 mg/l cefotaxime-treated and non-treated cultures of both genotypes. Addition of cefotaxime at 250 mg/l enhanced the regeneration frequency 12.5 and 10.5% of cv.capella and SWSR2, respectively, in relation to non-treated cultures. Although there was no obvious effect on the regeneration efficiency (defined as regeneration scores) at this concentration, but the regenerated shoots on this culture appeared healthy with dark green leaves.

The concentration of 500 mg/l cefotaxime in SEM2 increased the regeneration frequency of cv.capella to 64%, while the same concentration had no effect in improving the same studied characters in SWSR2 inbred line (Fig. 14 and Table 17).

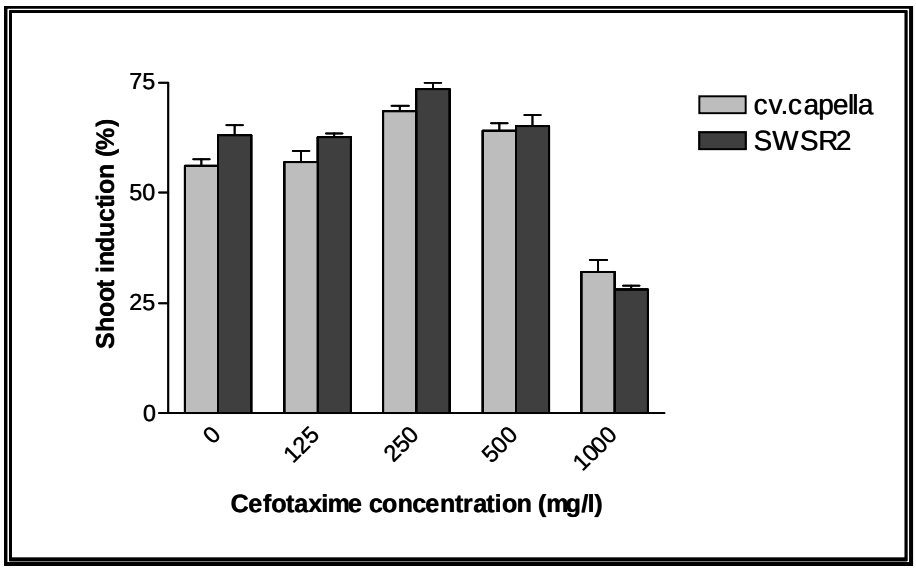

Fig. (14): Effect of different cefotaxime concentrations on regeneration frequency of split shoot apices from cv.capella and SWSR2 inbred line on SIM2. Data are means of four replicates ± SE.

Table (17): Regeneration efficiency of regenerated shoots from cv.capella and SWSR2 inbred line on SIM2 supplemented with different cefotaxime concentrations.

Concentration (mg/l)	Regeneration efficiency*	
	cv.capella	SWSR2
0	4.50 ± 0.34	4.68 ± 0.24
125	4.57 ± 0.37	4.64 ± 0.17
250	4.80 ± 0.13	4.70 ± 0.31
500	4.49 ± 0.21	4.30 ± 0.42
1000	1.27 ± 0.29	1.90 ± 0.35

*Regeneration efficiency was expressed as a score from 1 to 5. Values are means of four replicates ± SE.

The frequencies and efficiencies of shoot induction were dramatically decreased, reaching values of 32, 28% and 1.2, 1.9 of cv.capella and SWSR2 inbred line, respectively, by culturing explants on SEM2 containing 1000 mg/l cefotaxime (Fig. 14 and Table 17).

❖ ***Effect of carbenicillin on plant regeneration***

Using of 25 mg/l of carbenicillin in cultures of cv.capella increased the regeneration frequency from 59.5 to 67.5 % on the other hand, the regeneration efficiency decreased from 4.7 to 3.1. Conversely, a slight decrease was detected in the shoot induction frequency of SWSR2 inbred line in addition to the strong inhibition of regeneration efficiency (from 4.4 to 2.3) at the same concentration comparing with non-treated cultures (Fig. 15 and Table 18).

Generally, the drastic inhibition effect on the regeneration frequency started from 50 mg/l carbenicillin up to 200 mg/l, in which the regeneration percentage dropped to 19.5 and 25% for cv.capella and SWSR2 inbred line, respectively. Whereas, the same inhibition effect in the regeneration efficiency started at 25 mg/l carbenicillin. The similar results were observed in regeneration efficiency which recorded the lowest regeneration scores (1.7 and 1.4) of cv.capella and SWSR2 inbred line, respectively, on SEM2 supplemented with 200 mg/l carbenicillin.

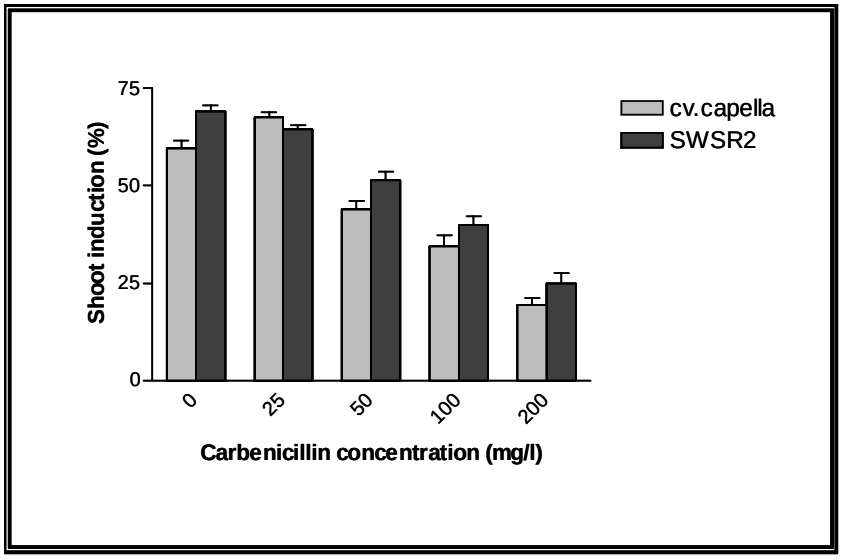

Fig. (15): Effect of different carbenicillin concentrations on regeneration frequency of split shoot apices from cv.capella and SWSR2 inbred line on SIM2. Data are means of four replicates ± SE.

Table (18): Regeneration efficiency of regenerated shoots from cv.capella and SWSR2 inbred line on SIM2 supplemented with different carbenicillin concentrations.

Concentration (mg/l)	Regeneration efficiency *	
	cv.capella	SWSR2
0	4.75 ± 0.21	4.49 ± 0.50
25	3.10 ± 0.29	2.33 ± 0.35
50	2.31 ± 0.11	2.11 ± 0.20
100	2.03± 0.25	1.80 ± 0.12
200	1.73 ± 0.27	1.47 ± 0.24

* Regeneration efficiency was expressed as a score from 1 to 5. Values are means of four replicates ± SE.

Moreover, starting from 50 mg/l carbenicillin the necrosis appeared on the regenerated shoots after two weeks of induction.

Therefore, the concentration of 250 mg/l cefotaxime was selected for the purpose of *Agrobacterium* elimination and at the same time enhanced the regeneration frequency and efficiency of treated explants.

2- Sensitivity to selective antibiotics

The first step to establish transformation protocol based on kananycin or hygromycin selection was to investigate the sensitivity of split shoot apices of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line to selective antibiotics. Non-transformed explants were cultured on SIM2 comprising different concentrations of kananamycin or hygromycin.

Although both antibiotics belong to the family of aminoglycoside antibiotics, they are structurally different. In addition; kanamycin is inactivated with neomycin phosphotransferase (NPTII) whereas, hygromycin inactivated with hygromycin

phosphotransferase (HPT). Influence of different antibiotic concentrations on shoot induction frequency was recorded in Fig. 16, 17 and 18.

❖ ***Effect of kanamycin on plant regeneration***

The regeneration frequency of *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line was decreased as the addition of kanamycin increased from 12.5 to 200 mg/l. Addition of 200 mg/l kanamycin to SIM2 prevented shoot induction incidence in both genotypes (Fig. 16).

Despite of the considerable amount of regeneration frequency at 50-100 mg/l, it was in pattern of initiation of shoot induction. The induced shoots stopped growing rapidly and failed to complete the growth in both genotypes. The most interesting observation was that the induced shoots on SIM2 supplemented with any kanamycin concentration were weak, had white leaves and died after a short time (Fig. 17).

In addition, the preliminarily transformation results also proved the steadily bad effect of kanamycin on plant regeneration (data not shown), thus kanamycin was found not to be a suitable marker for sunflower transformation.

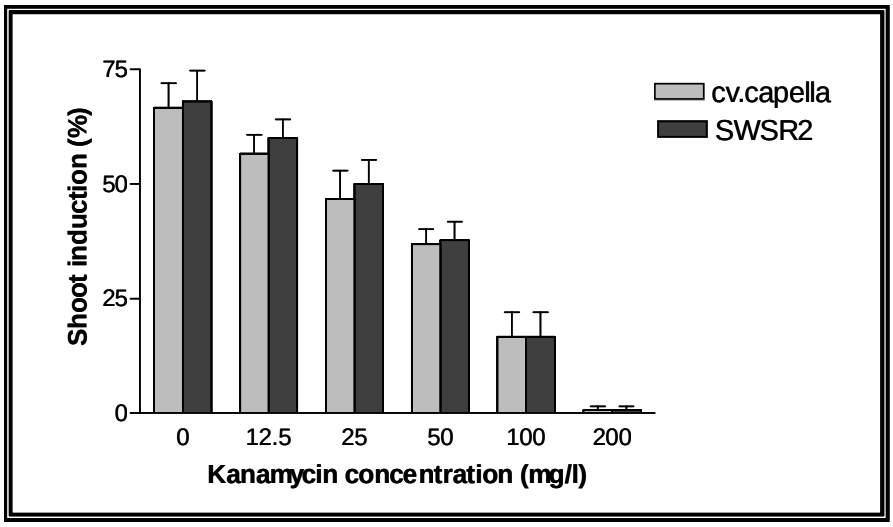

Fig. (16): Effect of different kanamycin concentrations on regeneration frequency of split shoot apices from cv.capella and SWSR2 inbred line, on shoot induction medium (SIM2). Data are means of five replicates ± SE.

Fig. (17): Effect of different kananycin and hygromycin concentrations on regeneration efficiency of split shoot apices of *H. annuus* L. genotypes, e.g. cv.capella on SIM2 supplemented with 12.5 mg/l kanamycin.

❖ ***Effect of hygromycin on plant regeneration***

With regard to Fig.18 appeared that hygromycin had a dramatically toxic effect on cultures even at a low concentration (5 mg/l). This effect was clearly observed at 20 and 40 mg/l.

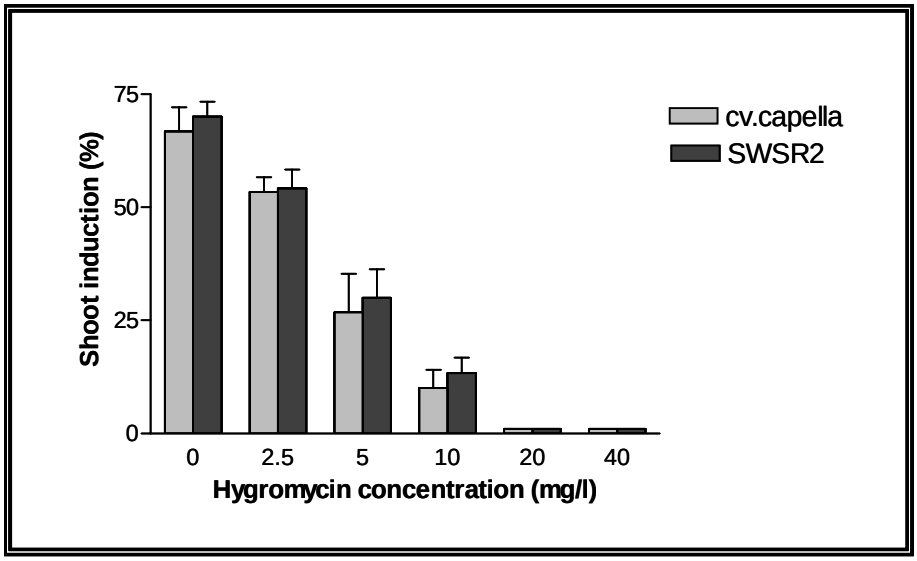

Fig. (18): Influence of various hygromycin concentrations on regeneration frequency of split shoot apices from cv.capella and SWSR2 inbred line on shoot induction medium (SIM2). Data are means of five replicates ± SE.

Culturing of split shoot apices on SEM2 containing 2.5 mg/l hygromycin reduced the shoot induction frequency approximately 13 and 16% in cv.capella and SWSR2 inbred line, respectively. Moreover, all shoot induction percentages presented initiation pattern of shoot induction with completely white leaves (Fig. 17). After 7-10 days of shoot induction the induced shoots died and turned brown (necrotic). Therefore, using of hygromycin was avoided in the transformation experiments as a selective antibiotic.

3- Test of GUS linearity

Fig. 19 revealed the time course analysis of fluorometric GUS activity of transformed and non transformed sunflower shoot apices. The GUS activity increased with the time, but the increase rate from 30 min. to 60 min. was higher than from 60 min. to 90 min. Therefore, the fluorometric GUS activity was measured after 60 min. in all transformation experiments because this time was found to be within the linearity of the GUS enzyme activity.

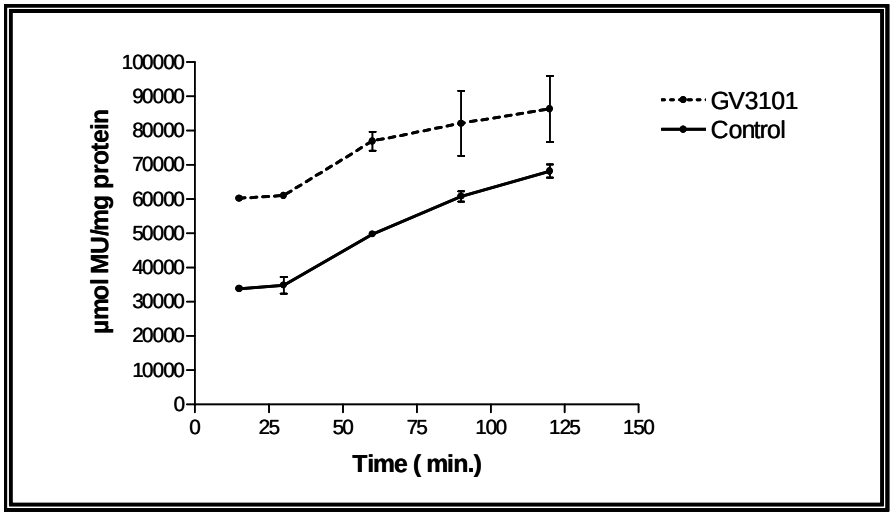

Fig. (19): Time course analysis of fluorometric GUS activity of transformed and non-transformed shoot apices of *H. annuus* L. Data are means of three replicates ± SE.

4- Effect of methanol on GUS activity

In the first transformation experiments, which tested the effect of different *Agrobacterium* strains, fluorometric GUS assay was performed with and without

addition of 20% methanol to the assay buffer to find out the effect of methanol on endogenous β- glucuronidase activity of transformed and non-transformed sunflower regenerated shoot as well as the fluorometric GUS activity using various bacterial strains.

As shown in Fig. 20 the addition of 20% methanol to the assay buffer decreased the fluorometric GUS activity of non transformed shoots approximately 38% in cv.capella, while this decrease represents about 30% in SWSR2 inbred line.

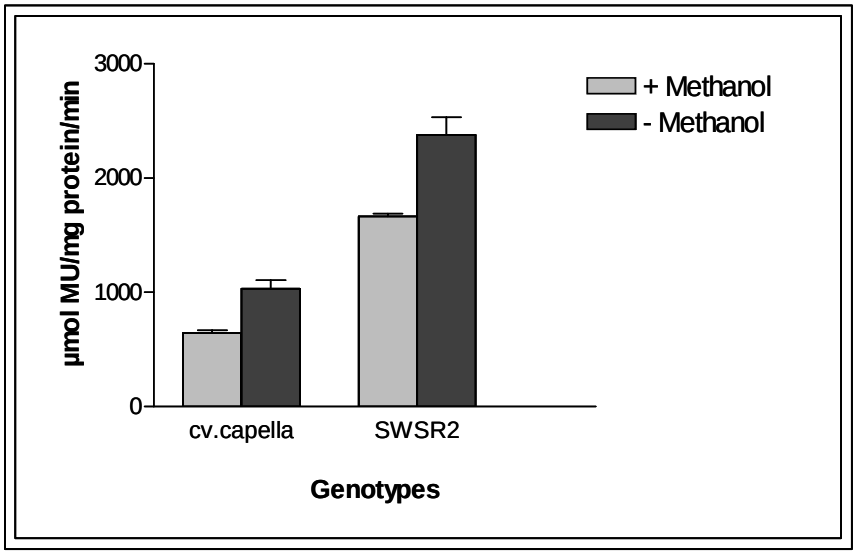

Fig. (20): Influence of methanol on the reduction of endogenous GUS like activity detected in non-transformed *H. annuus* L. shoot apices of cv.capella and SWSR2 inbred line. Data are means of fluorometric GUS assay measurements of five replicates ± SE.

Effect of methanol on the fluorometric GUS activity of transformed shoots using various bacterial strains was demonstrated in Fig. 21. The fluorometric GUS activity of transformed shoots varied among different tested bacterial strains in both genotypes.

Addition of 20% methanol increased the fluorometric GUS activity of GV3101 and C58 from 193.5 to 575.7 and from 240 to 382.6 µmol MU/mg protein /min., respectively, in cv.capella. In contrary, no improving effect was obtained when LBA4404 and EHA101were used (Fig. 21A).

In the case of SWSR2 inbred line, GV3101 and LBA4404 had achieved the highest fluorometric GUS activity values among all tested bacterial strains when 20% methanol was added to the assay buffer, while C58 and EHA101 strains appeared no stimulation effect on the fluorometric GUS activity under the same conditions (Fig. 21B)

Consequently, addition of 20% methanol to the assay buffer was applied for measurement the fluorometric and histochemical GUS activity for all transformation experiments.

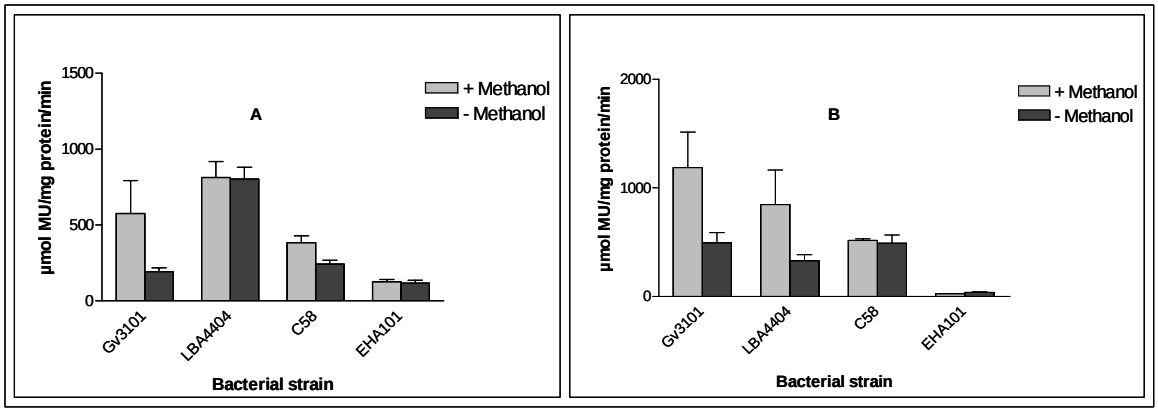

Fig. (21): Effect of methanol on the fluorometric GUS activity measurements of various tested bacterial strains: (A) cv.capella and (B) SWSR2 inbred line. Data are means of five replicates ± SE.

5- Transformation methods

On the basis of all the previous results, different transformation methods were compared. For each transformation method, several parameters were optimized in order to determine the most efficient transformation system.

5-1-*Agrobacterium* infiltration method

5.1.1- Effect of vacuum duration on split shoot apices transformation

These experiments were designed to investigate the effect of the durations of vacuum treatments on T-DNA delivery, represented as fluorometric and histochemical GUS activity, as well as regeneration frequency and efficiency and plant cell vitality (Fig. 22, 23 and Table 19).

The fluorometric results of these experiments were demonstrated in Fig. 22, which revealed that the fluorometric GUS activity increased 1.8 and 1.3 fold in cv.capella and SWSR2 inbred line, respectively, as the vacuum duration increased from 30 sec. to 60 sec. The increase was more obvious in cv.capella than in SWSR inbred line.

When vacuum duration exceeded than 60 sec., the fluorometric GUS activity started to decrease reaching the lowest values at 480 sec. (621.9 and 1325.2 µmol MU/mg protein/min.) from cv.capella and SWSR inbred line, respectively. It was also observed that increasing the duration than 60 sec. resulting in *Agrobacterium* growth around the expants after co-cultivation and this growth was more difficult to control at the resulting and subsequent stages.

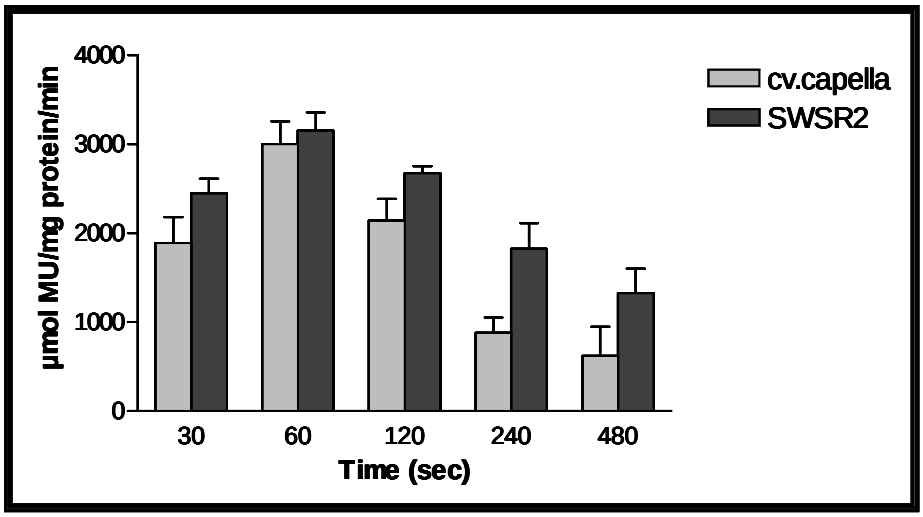

Fig. (22): Effect of different vacuum durations on the fluorometric GUS activity of two *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line. Values were recorded six to seven weeks after co-cultivation. Data are means of three replicates ± SE.

The similar results was observed in the histochemical GUS assay, which showed that the GUS expressing shoot frequency increased about 23 and 25% in cv.capella and SWSR inbred line, respectively, when the vacuum duration increased to 60 sec. No GUS expressing shoots were detected at 480 sec. in both genotypes (Table 19).

Fig. 23 presented the influence of different vacuum durations on the cell vitality, expressed as a mean of the yield (PAM 2000 fluorometer), of cv.capella and

SWSR2 inbred line. Generally, the cell vitality of the transformed shoots was inhibited with the increase of vacuum duration.

Application of vacuum at 150 mbar and a duration up to 60 sec. appeared a slightly effect on the cell vitality of the transformed shoots. The dramatically effect was detected at the relatively long vacuum durations (240-480 sec.)

These results were completed with the results of regeneration frequency and efficiency of the transformed explants (Table 19).

The regeneration frequency of both genotypes was slightly affected when vacuum duration increased up to 60 sec. The highest regeneration frequency was 63.3 and 66.7% from cv.capella and SWSR inbred line, respectively, at 30 sec. of vacuum. Conversely, the lowest regeneration percentage was 36.7and 33.3% from cv.capella and SWSR inbred line, respectively, at the longest vacuum duration

These results were confirmed with data of regeneration efficiency, which proved that the shortest vacuum duration the highest regeneration efficiency in relation to untreated explants (Table 19).

In conclusion, vacuum duration of 60 sec. for two times was applied in *Agrobacterium* infiltration methods as a compromise between the GUS activity and the cell vitality.

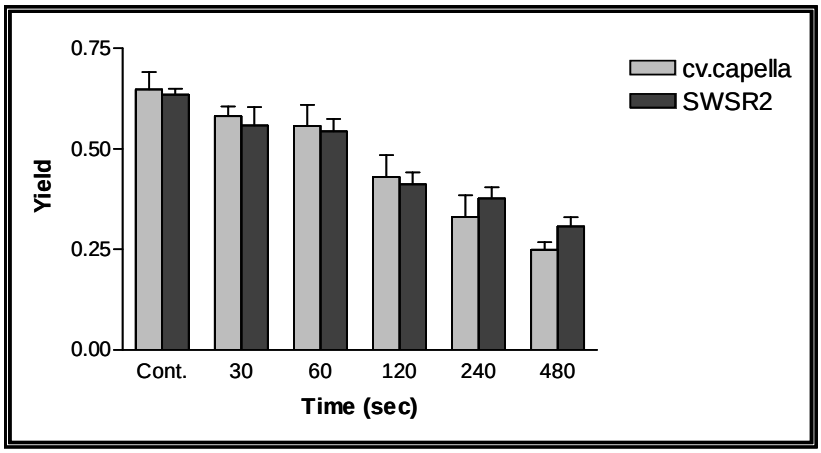

Fig. (23): Influence of different vacuum durations on the cell vitality of cv.capella and SWSR2 inbred line. The cell vitality was measured six to seven weeks after co-cultivation and expressed as a mean of the yield (PAM 2000 fluorometer) for three replicates ± SE.

Table (19): Effect of different durations of *Agrobacterium* infiltration on the regeneration frequency and efficiency and histochemical GUS assay of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Agrobacterium infiltration duration (sec.)	Regeneration % mean ± SE		Regeneration efficiency* mean ± SE		GUS expressing shoots %	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
0	66.7 ± 3.3	70.0 ± 0.0	4.6 ± 0.19	4.5 ± 0.30	0	0
30	63.3 ± 3.3	66.7 ± 6.6	4.6 ± 0.11	4.3 ± 0.09	10.5	10
60	60.0 ± 5.7	60.0 ± 0.0	4.3 ± 0.09	4.2 ± 0.29	33.3	35
120	50.0 ± 0.0	50.0 ± 5.7	2.9 ± 0.17	2.5 ± 0.10	6.7	13.3
240	40.0 ± 5.7	36.7 ± 3.3	2.2 ± 0.16	2.1 ± 0.13	0	9.0
480	36.7 ± 3.3	33.3 ± 8.8	1.5 ± 0.29	1.7 ± 0.17	0	0

* Regeneration efficiency was expressed as a score from 1 to 5. Means are values of three replicates.

5.1.2- Optimization of parameters enhancing *Agrobacterium* infiltration method of *H. annuus* L. shoot apices

In the present experiments the focus was placed on the optimization and evaluation of some transformation parameters aiming more efficient transformation of meristematic shoot apices of the two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line. Such parameters includ: bacterial strains (GV3101, LBA4404, C58 and EHA101), density of bacterial culture (OD_{600} 0.5, 1.0, 1.5 and 2.0), explant characteristics (split and intact shoot apices), co-cultivation media (MS and YEB), virulence inducer (200 µM acetosyringone and 100 µM coniferyl alcohol), co-cultivation duration (2 and 3 days) and pre-culture period (0, 1, 2 and 3 days). Parameters were tested, one at a time in a sequential order (as mentioned above) and each optimized parameter was applied to the subsequent experiment. These Parameters were evaluated on the basis of histochemical and fluorometric GUS activity coupled

with regeneration frequency and efficiency and plant cell vitality. Results were summarized in Table 20, 21 and Fig. 24, 25.

❖ ***Sensitivity of tested genotypes to variant bacterial strains.***

All the different tested bacterial strains produced transformation events with different efficiency (Table 20). LBA4404 and GV3101, carrying both the plasmid pBI121, were the most efficient strains with both genotypes.

Based on the percentage and the intensity of the fluorometric and histochemical assay after four weeks, LBA4404 strain (octopine group) was superior and more effective than the other strains on cv.capella and GV3101 strain (nopaline group) with SWSR2 inbred line (Table 20 and Fig. 24a).

The other nopaline strain tested in this study, C58, gave low fluorometric GUS activity values (382.7 and 518.1 µmol MU/mg protein/min.) with cv.capella and SWSR2 inbred line, respectively. This strain harbored the plasmid pAM194 and the reporter *gus* gene under the control of mas promoter. This means that the GV3101 nopaline group and 35S promoter, widespread used in plant transformation, was the most effective for SWSR2 inbred line.

Generally, comparing the percentage of the fluorometric assay after two and four weeks showed a reduction in the GUS activity with the time. This reduction represents 50% in cv.capella using GV3101 strain and the corresponding reduction recorded in SWSR2 inbred line was 30%. This indicates that the *gus* expression after two weeks was partially transient (Table 20).

The expression of the *gus* gene showed variability in the number of *gus* positive shoots and expression levels. By using of EHA101 strain, no GUS expressing shoots were detected in SWSR2 inbred line, while the percentage of transformed shoots of cv.capella was 20% with faint expression (Table 20).

With regard to the effect of using the previous *Agrobacterium* strains on regeneration frequency (regeneration percentage) and efficiency as well as the vitality of the transformed shoots, they had a slight effect on the shoot vitality and on the regeneration percentage, which ranged from 60-50% and 55-50% in cv.capella and SWSR2, respectively, (Fig. 24b, and Table 20). A relatively reduction in the regeneration efficiency appeared in both genotypes when EHA101 strain was used.

Table (20): Effect of different bacterial strains on the regeneration percentage and efficiency, fluorometric and histochemical GUS assay of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Bacterial strain	Regeneration % mean ± SE		% pos. Fluorometric assay (two weeks after co-cultivation)		% pos. Fluorometric assay (four weeks after co-cultivation)		% pos. Histochemical assay (four weeks after co-cultivation)		Regeneration efficiency [A] mean ± SE	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
Untreated	60 ± 0.0	55 ± 9.5	-	-	-	-	-	-	4.5 ± 0.29	3.9 ± 0.08
GV3101	55 ± 5.0	55 ± 5.0	80	70	30	40	40	50	4.3 ± 0.14	3.1 ± 0.11
LBA4404	55 ± 5.0	50 ± 5.7	80	60	40	30	50	30	4.3 ± 0.14	3.5 ± 0.61
C58	50 ± 5.7	50 ± 10.0	60	30	20	10	30	20*	4.1 ± 0.18	3.0 ± 0.35
EHA101	50 ± 5.7	50 ± 5.7	40	20	10	10	20*	nd	3.8 ± 0.22	2.8 ± 0.40

(*) light blue, pos.= positive, (nd) not detected and ([A]) regeneration efficiency was expressed as a score from 1 to 5. Means are values of four replicates.

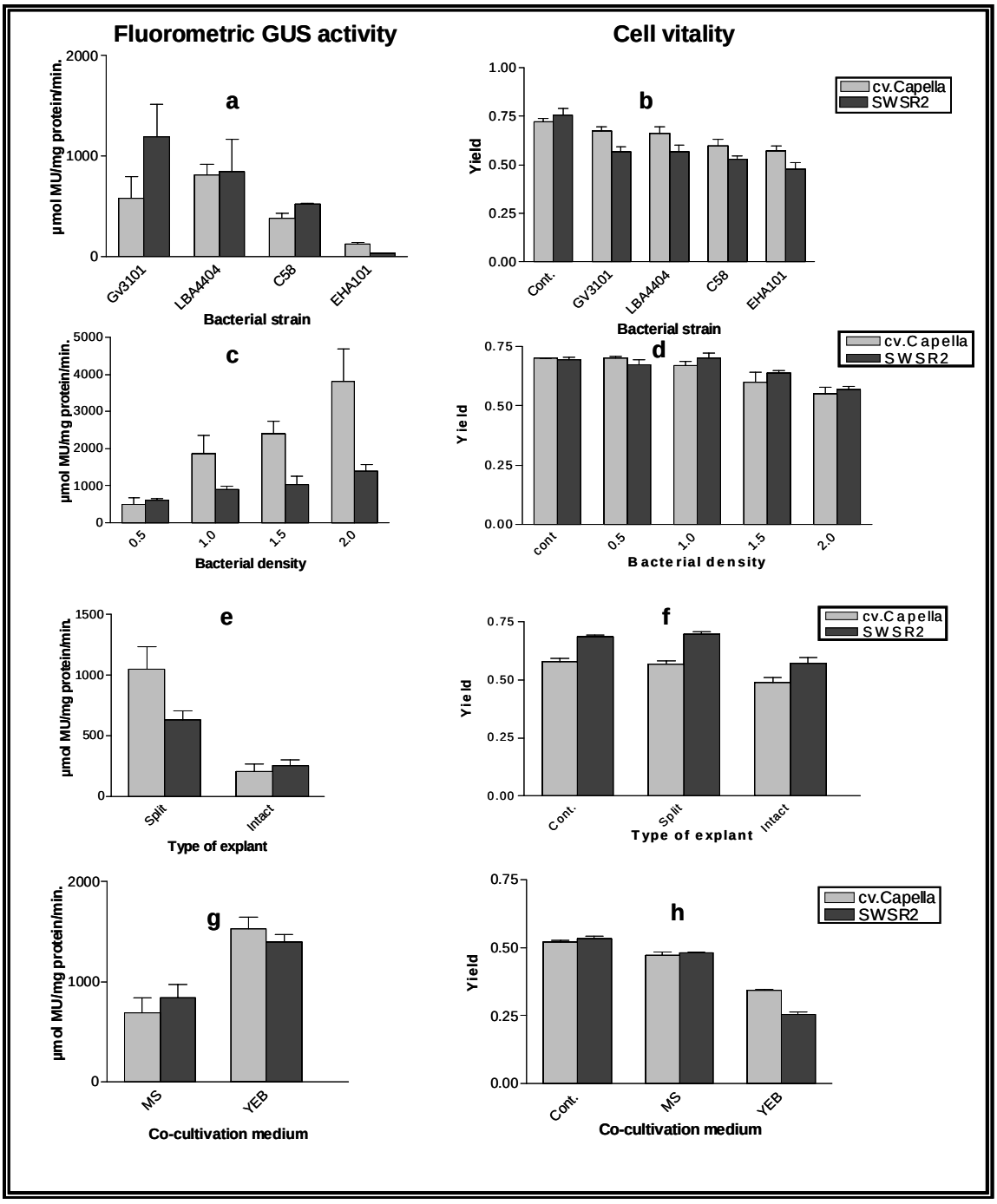

Fig. (24): Assessment of different parameters enhancing the *Agrobacterium* mediated transformation efficiency of split shoot apices for two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line on the basis of fluorometric GUS activity and the cell vitality. a,b: different bacterial strains, c,d: different bacterial densities, e,f: different types of explants, g,h: different co-cultivation media. Results are means of at least three replicates ± SE, the vitality was expressed as a mean of the yield (PAM 2000 fluorometer). Cont. = control.

Therefore, the strain LBA4404 was selected for cv.capella and GV3101 for SWSR2 inbred line for all the following transformation experiments.

❖ ***Effect of bacterial density***

The influence of different bacterial densities (OD_{600} 0.5, 1.0, 1.5 and 2.0) on the regeneration frequency, vitality, fluorometric and histochemical GUS activity was evaluated and recorded in Table 21 and Fig. 24c, d.

Histochemical and fluorometric GUS activity generally increased in both genotypes as the bacterial concentration increased (Table 21 and Fig. 24c). At OD_{600} value 2.0, the fluorometric GUS activity increased 7.6 and 2.3 fold in cv.capella and SWSR2 inbred line, respectively, comparing with the values of fluorometric GUS activity at OD_{600} value 0.5.

There was an inversely proportion between bacterial density and regeneration frequency and efficiency as well as cell vitality. Using the OD_{600} at value 2.0 decreased the regeneration percentage from 60 to 40% and 55 to 35% in cv.capella and SWSR2 inbred line, respectively, compared with the control. The highest regeneration efficiency was 4.4 and 4.6 using OD_{600} value 0.5 whereas, the lowest recorded score was 2.7 and 3.3 at OD_{600} value 2.0 in cv.capella and SWSR2 inbred line, respectively (Table 21). The same effect was observed on the cell vitality (Fig. 24d)

In conclusion, OD_{600} value 1.0 has been selected for subsequent transformation experiments as the suitable bacterial density for transformation without affecting on the cell vitality or regeneration characters in both genotypes.

❖ ***Effect of type of explant***

In the present study two types of shoot apices (intact and split) from cv.capella and SWSR2 inbred line were compared. Results in Table 21 and Fig. 24 showed that there was an increase in the GUS activity with the use of split explants.

The histochemical assay results showed that using split shoot apices increased the percentage of GUS expressing shoots 20% in both genotypes (Table 21). Similarly, the fluorometric values raised up to 1052.5 and 630 µmol MU/mg protein/min. in cv.capella and SWSR2 inbred line, respectively (Fig. 24e).

Meanwhile, the regeneration percentage was improved by longitudinally cutting of the explants and subsequent gene transfer.

Despite of the longitudinal section through the apical meristem favored multiple shoot induction, the regeneration efficiency of the regenerated shoots was higher than those from intact explants (Table 21). Similar results were obtained from cell vitality (Fig. 24f).

❖ ***Effect of co-cultivation media***

The effect of MS, which has been often used as a standard medium for transformation experiments, and YEB media on the GUS activity and the cell vitality, is illustrated in (Table 21 and Fig 24g, h). The use of YEB as a co-cultivation medium increased the fluorometric GUS activity 2.2 and 1.7 fold for cv.capella and SWSR2 inbred line, respectively compared with values of using MS as a co-cultivation medium (Fig. 24g). YEB as a co-cultivation medium had a negative effect on the vitality, regeneration frequency and efficiency. The reduction in the regeneration frequency represents about 20 and 15% in cv.capella and SWSR2 inbred line, respectively, in relation to untreated cultures. The lowest cell vitality values were registered from cv.capella and SWSR2 inbred line were 0.34 and 0.25, respectively, when YEB medium was used.

Thus, MS medium was chosen as a co-cultivation medium as a compromise between transformation efficiency, expressed as fluorometric and histochemical GUS activity, and the cell vitality.

❖ ***Effect virulence inducer***

Two virulence inducers (acetosyringone and coniferyl alcohol) were tested in an attempt to increase the infection probability of *Agrobacterium* for the explants.

The addition of 200 µM acetosyringone during the co-cultivation increased the fluorometric GUS activity 2.8 fold in both genotypes, while addition of 100 µM coniferyl alcohol increased the fluorometric GUS activity 1.2 and 1.3 fold in cv.capella and SWSR2 inbred line, respectively, comparing with the transformed without inducer (Fig. 25i). These results were also confirmed with the histochemical GUS assay results.

Addition of 200 µM acetosyringone recorded the highest GUS expressing shoot percentage (50 and 40%) from cv.capella and SWSR2 inbred line, respectively.

However, addition of the virulence inducers had no effect on the regeneration percentage and efficiency in both genotypes in compared to untreated explants (Table 21).

According to the present results, acetosyringone was added to the *Agrobacterium* suspension during its virulence induction period in all consequent experiments.

❖ ***Effect of pre-culture and co-cultivation duration***

Prior to inoculation with *Agrobacterium*, explants were pre-cultured on SIM2 medium for 0 day to 3 days. The presented results in Table 21 and Fig. 25l, m revealed that *gus* expression as well as fluorometric GUS activity increased with length of pre-culture duration. Similarly, regeneration percentage and efficiency and shoot vitality were enhanced with increasing the pre-culture period.

Pre-culture the explants on SEM2 for 3 days raised the histochemical and fluorometric values up to 40% and 1945.6 µmol MU/mg protein/min., in cv.capella, while the corresponding values recorded from SWSR2 inbred line were 30% and 1514.4 µmol MU/mg protein/min., respectively (Table 21 and Fig 25l).

Explants showed a hypersensitivity response to the bacterial infection without any pre-culture. The lowest cell vitality value (expressed as a yield) was 0.42 and 0.40 from cv.capella and SWSR2 inbred line, respectively, when the explants were directly transformed (Fig. 25m). A short pre-culture period (1-3 days) increased the regeneration percentage as well as the regeneration efficiency. Culturing the explants for 3 days before transformation incidence increased the regeneration frequency 15% in both genotypes in compare to the regeneration frequency of the explants without pre-culture

After pre-culture, the explants were transformed by co-cultivation for two and three days with *Agrobacterium*. A slight or no effect was observed on the regeneration percentage and efficiency and shoot vitality resulted from using different co-cultivation durations (Table 21 and Fig. 25k). Co-cultivating the explants for three days increased the fluorometric GUS activity 4.6 and 2.5 fold in cv.capella and SWSR2 inbred line,

respectively, relative to the co-cultivation for two days Theses data were also proved by histochemical data (Table 21 and Fig. 25j).

Finally, pre-culturing the explants on the shoot induction medium for three days followed by co-cultivation with *Agrobacterium* for three days was found to be the best conditions in the established transformation protocol.

In addition, in most transformation experiments the transformation efficiency, expressed as fluorometric GUS activity, of the hybrid (cv.capella) was higher than the inbred line (SWSR2).

5.1.3- Estimation of transformation frequency

In this transformation experiment, all the resulting optimal parameters were applied on split shoot apices of cv.capella and SWSR2 inbred line. After co-cultivation duration, explants were washed in liquid shoot induction medium 2 containing 250 mg/l cefotaxime, blotted on sterile filter paper and cultured on solid SIM2 supplemented with 250 mg/l cefotaxime to eliminate the *Agrobacterium* without using selection agents. For further growth regenerated shoots were transferred to root induction media 1, then shoot elongation and root development medium 2. Plants were subjected to histochemical, fluorometric and molecular analysis. The obtained results were summarized in (Table 22 and Fig 26, 27).

❖ ***Histochemical GUS assay***

Histochemical GUS assay results documented that the transformed tissues were uniformly expressed for GUS staining, in contrary to untreated (control) tissues which did not exhibit any type of blue color (see Fig. 44). The GUS frequency reached 4 and 3% in cv.capella and SWSR2 inbred line, respectively, in relation to the total number of co-cultivated explants (Table 22).

❖ ***Fluorometric GUS assay***

The fluorometric values revealed that the hybrid had a superior response than the inbred line and achieved 1954.6 µmol MU/mg protein/min. whereas, the corresponding value was 1524.4 µmol MU/mg protein/min. of the inbred line (Table 22).

❖ ***PCR analysis of transformants***

Three various DNA extraction protocols were tested and results compared concerning DNA yield quality, quantity and labour intensity. the first one (modified after Dellaporta *et al*., 1983) was a so-called fast protocol with a high throughput of samples and low costs, but the obtained DNA was not enough even for PCR analysis. The second protocol, *DNeasy plant Mini Kit* which was also fast and resulted in quality and quantity of DNA which was appropriate for PCR analysis but not useful when high molecular weight DNA is required. The third one was CTAB protocol which yielded high quality and quantity of genomic DNA appropriate for PCR and southern blot analysis.

Thus, the CTAB DNA extraction protocol was used for all transformation experiments of this study.

As a confirmation of the presence of the *gus* gene in the *gus* positive plants. *EcoRI*-digested genomic DNA of independently random transformed and non-transformed (control) tissues were analyzed using primers specific to *gus* and *nptII* gene. The expected 830-bp band was amplified in the GUS expressing tissues from *H. annuus* L. cv.capella and SWSR2 inbred line whereas, no amplification was detected in the non-transformed tissues (Fig. 26). Out of 30 tested PCR plants only 4 and 3 plants were positive with both primers of cv.capella and SWSR2 inbred line, respectively.

In order to test the possibility of bacterial contamination of the plant tissue, *virA* specific primer was used in an additional PCR test. No amplification was detected in any of the transgenic material analyzed (Fig. 27). This indicates that no residual *Agrobacterium* was present in the analyzed material.

Transformation frequency was calculated on the basis of positive PCR plants and recorded as a percentage of the total number of co-cultivated explants. This transformation frequency was 4 and 3% of cv.capella and SWSR2 inbred line, respectively (Table 22).

Table (21): Effect of different *Agrobacterium* mediated transformation parameters on the regeneration percentage and efficiency and histochemical GUS assay of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Transformation parameters	Regeneration % mean ± SE		Regeneration efficiency [A] mean ± SE		GUS expressing shoots %	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
Bacterial density OD_{600}						
Untreated	60 ± 8.2	55± 4.0	4.5 ± 0.23	4.6 ± 0.07	0	0
0.5	55 ± 5.0	55 ± 5.0	4.4 ± 0.20	4.6 ± 0.16	0	0
1.0*	55 ± 5.0	50 ± 0.0	4.1 ± 0.12	4.3 ± 0.17	30	30
1.5	50 ± 0.0	45 ± 2.8	3.4 ± 0.20	3.8 ± 0.09	40	30
2.0	40 ± 4.0	35 ± 5.0	2.7 ± 0.25	3.3 ± 0.20	40	40
Type of explant						
Untreated	60 ± 8.1	55 ± 2.8	4.3 ± 0.15	4.6 ± 0.08	0	0
Split*	60 ± 4.1	55 ± 2.8	4.0 ± 0.17	4.3 ± 0.19	30	30
Intact	50 ± 4.0	50 ± 0.0	3.7 ± 0.20	3.8 ± 0.22	10	10
Co-cultivation medium						
Untreated	60 ± 5.7	55 ± 6.4	4.5 ± 0.15	4.3 ± 0.20	0	0
MS*	60 ± 10.0	55 ± 2.8	4.3 ± 0.06	4.0 ± 0.17	30	20
YEB	40 ± 5.7	40 ± 4.0	2.6 ± 0.17	2.6 ± 0.22	20	30
Virulence inducer						
Untreated	60 ± 4.0	55± 2.8	nd	nd	0	0
Without	55 ± 2.8	60 ± 4.0	nd	nd	20	10
100µM coniferyl alcohol	55 ± 6.4	50 ± 0.0	nd	nd	10	10
200µM acetosyringone*	60 ± 8.1	55 ± 6.4	nd	nd	50	40
Co-cultivation duration						
Untreated	60 ± 8.1	50 ± 4.0	4.3 ± 0.19	4.5 ± 0.10	0	0
2 days	55 ± 6.4	55 ± 2.8	4.3 ± 0.08	4.4 ± 0.06	10	10
3 days*	55 ± 6.5	55 ± 6.4	4.0 ± 0.29	4.3 ± 0.27	40	30
Pre-culture period						
Untreated	60 ± 0.0	55 ± 9.5	4.8 ± 0.06	4.5 ± 0.19	0	0
0 day	45 ± 5.0	40 ± 0.0	2.3 ± 0.10	2.0 ± 0.09	0	0
1 day	55 ± 5.0	50 ± 5.7	3.0 ± 0.08	3.0 ± 0.15	10	0
2 days	55 ± 5.0	50 ± 5.8	3.5 ± 0.17	3.8 ± 0.16	20	20
3days*	60 ± 0.0	55 ± 5.0	4.5 ± 0.29	4.3 ± 0.22	40	30

(*) Chosen condition for subsequent experiments, (nd) not determined, ([A]) regeneration efficiency was expressed as a score from 1 to 5. Means are values of at least three replicates.

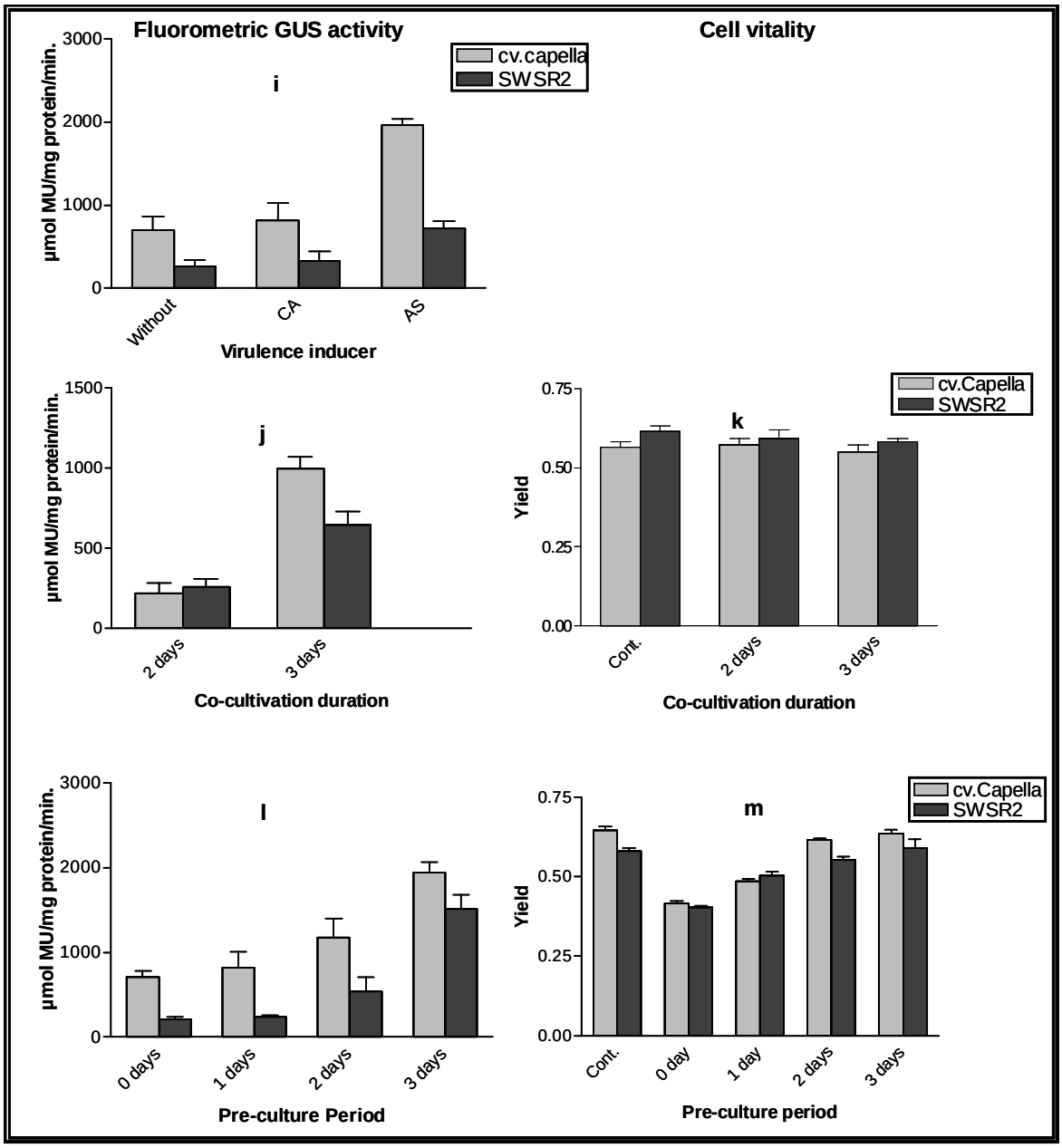

Fig.(25): Assessment of different parameters enhancing the *Agrobacterium* mediated transformation efficiency of split shoot apices for two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line based on fluorometric GUS activity and the cell vitality. i: different inducers, j,k: different co-cultivation durations and l,m: different pre-culture periods. Results are means of at least three replicates ± SE. the vitality was expressed as a mean of the yield (PAM 2000 fluorometer). Cont. = control, CA=coniferyl alcohol and AS= acetosyringone.

Table (22): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using *Agrobacterium* infiltration method.

Genotype	Total No of co-cultivated explants	No of + ve Gus plants	Gus frequency* (%)	Fluorometric assay (µmol MU/mg protein/min.) Mean + SE	No of tested PCR plants	No of + ve PCR plants	Transformation frequency* (%)
cv.capella	100	4	4	1954.6±118.4	30	4	4
SWSR2	100	3	3	1524.4±166.6	30	3	3

(*) The percentage was calculated in relation to the total number of co-cultivated explants.
PCR was performed with *gus* and *nptII* primers after 14-16 weeks after co-cultivation. Transformation frequency was calculated on the basis of positive PCR plants.

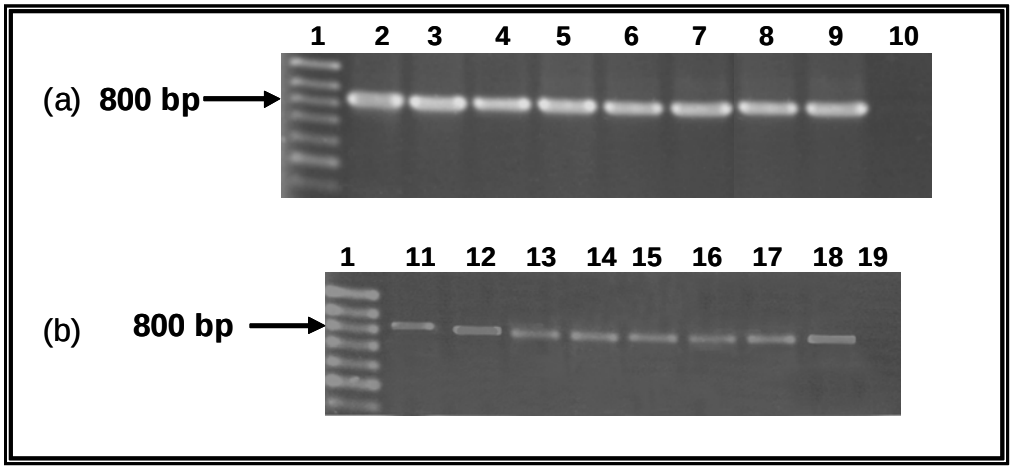

Fig. (26): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2-4 and 11-13) transformed SWSR2 inbred line plants, lane (5, 14) positive control (pure plasmid), lane (6-9 and 15-18) transformed cv.capella plants, and lane (10, 19) negative control (untreated plants).

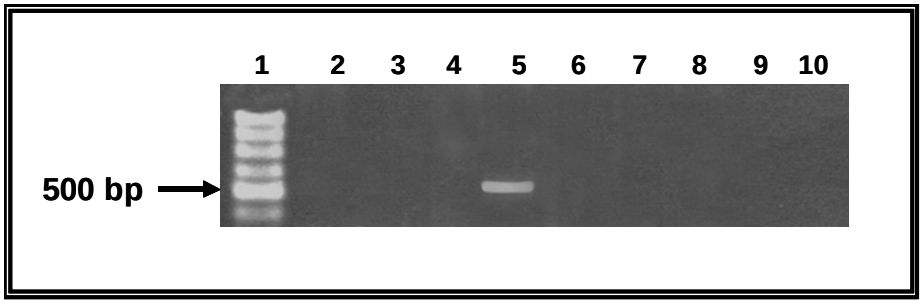

Fig. (27): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primer to *virA* gene. Lane (1) molecular marker DNA, lane (2-4) transformed SWSR2 inbred line plants, lane (5) positive control (*Agrobacterium* DNA), lane (6-9) transformed cv.capella plants, and lane (10) negative control (untreated plants).

5.2- Wounding strategies and *Agrobacterium* infiltration method

In these transformation experiments, microprojectiles and glass beads were used as two different wounding strategies combined with *Agrobacterium* infiltration method (all the optimized parameters except pre-culture period) in an attempt to increase the transformation frequency with minimal interference with the regeneration potential. Evaluation of these conditions was based on histochemical and fluorometric GUS activity coupled with regeneration percentage and efficiency and plant cell vitality.

5.2.1- Using particle bombardment for wounding the tissue

a- Assessment of different physical factors

Two physical factors (particle size and particle acceleration pressure) were studied in an attempt to optimize the wounding conditions that increase the transformation frequency with minimal tissue damage. Results of using the combination of different uncoated particle sizes (0.4 and 0.7 µm) with various particle acceleration pressures (450, 900, 1550 and 1800 psi) were illustrated in Fig. 28, 29 and Table 23.

The fluorometric results of cv.capella showed that the transformation events generally increased as the particle acceleration pressure (helium pressure) increased up

to 1550 psi using either 0.4 or 0.7 µm particles. Reversely, when the helium pressure exceeded than 1550 psi, the fluorometric values dramatically decreased reaching 134.0 and 87.3 µmol MU/mg protein/min. at 1800 psi with using 0.4 and 0.7 µm particles, respectively. Increasing the helium pressure to 1550 psi raised the fluorometric GUS activity 5.9 and 2.3 fold with using of 0.4 and 0.7 µm particles, respectively, compared to 450 psi (Fig. 28A).

The similar results were obtained from the histochemical assay which achieved the highest *gus* expression frequency at 1550 psi with 0.4 µm particles (Table 23).

As far as the cell vitality is concerned, all the used helium pressures steadily reduced the vitality of the plant cells to different levels. This reduction was clearly appeared by using 0.7 µm than 0.4 µm particles (Fig 28 B). The regeneration frequency as well as the efficiency was also highly affected by different helium pressures used (Table 23). Thus, the combination of 1550 psi and 0.4 µm particles was found to be a compromise between cell vitality and transformation frequency, expressed as fluorometric GUS activity.

Fig. 29 showed the effect of using different helium pressures and particle sizes on the fluorometric GUS activity as well as the vitality of the plant cells in SWSR2 inbred line. The general pattern of response was as in cv.capella with respect to particle size. Using 1550 psi helium pressure and 0.7 µm uncoated particles yielded the highest fluorometric GUS activity (1650.7 µmol MU/mg protein/min.).

The fluorometric results were confirmed with histochemical GUS assay results (Table 23). Using the 0.7 µm particles at 1550 psi helium pressure increased the *gus* expression frequency about 14% than using the same size of particles at 450 psi

In terms of regeneration frequency and efficiency, the higher helium pressures the lower regeneration frequencies and efficiencies. The lowest regeneration frequency and efficiency recorded from SWSR2 were 10% and 1.7 regeneration score, respectively, when 0.7 µm particles at 1800 psi were used (Table 23). In addition, at a pressure of 1800 psi with either 0.4 or 0.7 µm particles size, more than 90% of the bombarded tissues of both genotypes became necrotic and formation of calli was observed.

The same manner of response was observed in the influence of the studied parameters on the plant cell vitality of SWSR2 inbred line (Fig. 29B).

Overall, the transformation efficiency (expressed as fluorometric GUS activity) of the inbred line was higher than the hybrid using this method of transformation.

Therefore, the combination of 1550 psi with 0.4 and 0.7 µm particles was selected for estimating the transformation frequency of this method for cv.capella and SWSR2, respectively.

b- Estimation of transformation frequency

In this transformation experiment, all the resulting optimal parameters were applied on split shoot apices of cv.capella and SWSR2 inbred line. Plants were subjected to histochemical, fluorometric and molecular analysis and the obtained results were presented in Table 24 and Fig. 30, 31.

❖ ***Histochemical GUS assay***

The recorded *gus* expression frequency was 2 and 3.3% in cv.capella and SWSR2 inbred line, respectively, in relation to the total number of co-cultivated explants (Table 24). Moreover, *gus* expression was completely and uniformly in the transformed tissues, while no expression was detected in the untreated tissues (control) (See Fig., 44).

❖ ***Fluorometric GUS assay***

The fluorometric results showed that the inbred line had a superior response than the hybrid and achieved 1660.8 µmol MU/mg protein/min, while the corresponding value of the hybrid was 1041.6 µmol MU/mg protein/min. (Table 24).

❖ ***PCR analysis of transformants***

The expected 830-bp band was amplified in the *gus* expressing tissues from *H. annuus* L. cv.capella and SWSR2 inbred line, whereas no amplification was detected in the non-transformed tissues. Out of 25 tested PCR plants only 2 and 3 plants were positive with both primers of cv.capella and SWSR2 inbred line, respectively (Fig. 30). There was no *Agrobacterium* contamination in the positive plants (Fig. 31). The transformation frequency of cv.capella was 2%, while the corresponding frequency of SWSR2 was 3.3%

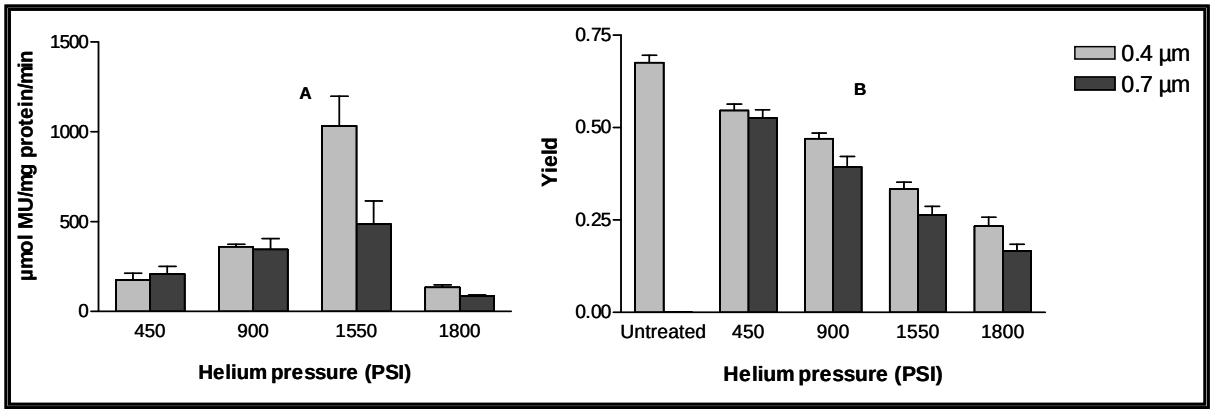

Fig. (28): Evaluation of different wounding parameters using particle bombardment coupled with *Agrobacterium* infiltration method of split shoot apices for high oleic *H. annuus* L. cv.capella using fluorometric GUS activity and the cell vitality. A: fluorometric GUS activity and B: cell vitality. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer) and results are means of four replicates ± SE.

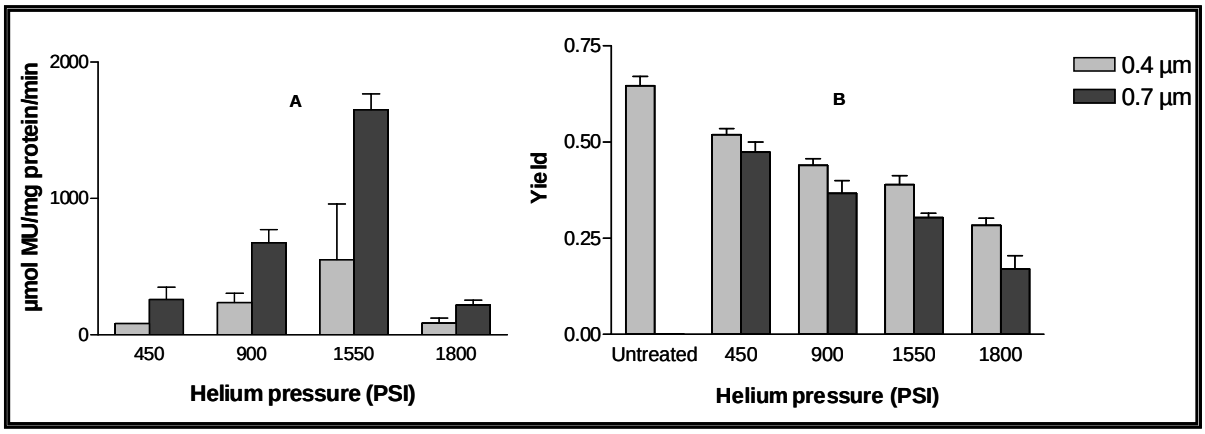

Fig. (29): Evaluation of different wounding parameters using particle bombardment coupled with *Agrobacterium* infiltration method of split shoot apices for high oleic *H. annuus* L. SWSR2 inbred line using fluorometric GUS activity and the cell vitality. A: fluorometric GUS activity and B: cell vitality. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer) and results are means of four replicates ± SE.

Table (23): Effect of different wounding parameters using particle bombardment on the regeneration percentage and efficiency and histochemical GUS assay of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Wounding parameters	Regeneration % mean ± SE		Regeneration efficiency [A] mean ± SE		GUS expressing shoots %	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
Untreated	67.5 ± 4.7	70.0 ± 4.0	4.8 ± 0.04	4.6 ± 0.10	0.0	0.0
Particle size (µm) **0.4**						
Helium pressure (PSI)						
450	55.0 ± 2.8	65.0 ± 2.8	4.0 ± 0.06	4.1 ± 0.08	0.0	0.0
900	47.5 ± 2.5	57.5 ± 2.5	3.2 ± 0.08	3.2 ± 0.18	9.10	12.5
1550	35.0 ± 2.8	47.5 ± 2.5	2.7 ± 0.20	2.4 ± 0.16	28.6	14.3
1800	20.0 ± 4.0	25.0 ± 5.0	2.3 ± 0.14	2.0 ± 0.20	11.1	8.30
Particle size (µm) **0.7**						
Helium pressure (PSI)						
450	52.5 ± 4.7	55.0 ± 5.0	3.7 ± 0.12	3.8 ± 0.06	0.0	6.3
900	37.5 ± 2.5	50.0 ± 4.0	3.0 ± 0.14	3.0 ± 0.18	12.5	7.1
1550	25.0 ± 2.8	35.0 ± 2.8	2.3 ± 0.10	2.1 ± 0.22	18.2	20
1800	7.50 ± 2.5	10.0 ± 4.0	1.9 ± 0.16	1.7 ± 0.20	0.0	16.6

Explants were bombarded twice using tungsten particles and 6 cm distance between macrocarrier assembly and target plate. Mean are values of four to five replicates. ([A]) regeneration efficiency was expressed as a score from 1 to 5.

Table (24): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using microprojectiles wounding system combined with *Agrobacterium* infiltration method.

Genotype	Total No of co-cultivated explants	No of + ve Gus plants	Gus frequency* (%)	Fluorometric assay (µmol MU/mg protein/min.) Mean + SE	No of tested PCR plants	No of + ve PCR plants	Transformation frequency* (%)
cv.capella	100	2	2	1041.6±164.0	25	2	2
SWSR2	90	3	3.3	1660.8±114.3	25	3	3.3

(*) The percentage was calculated in relation to the total number of co-cultivated explants and PCR was performed with *gus* and *nptII* primers 14-16 weeks after co-cultivation. Transformation frequency was calculated on the basis of positive PCR plants.

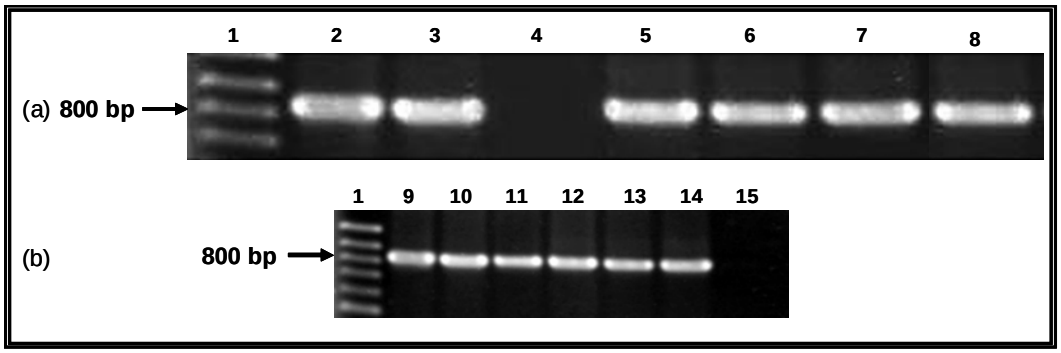

Fig. (30): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2, 3 and 9, 10) transformed cv.capella plants, lane (4, 15) negative control (untreated plants), lane (5, 14) positive control (pure plasmid) and lane (6-8 and 11-13) transformed SWSR2 inbred line plants.

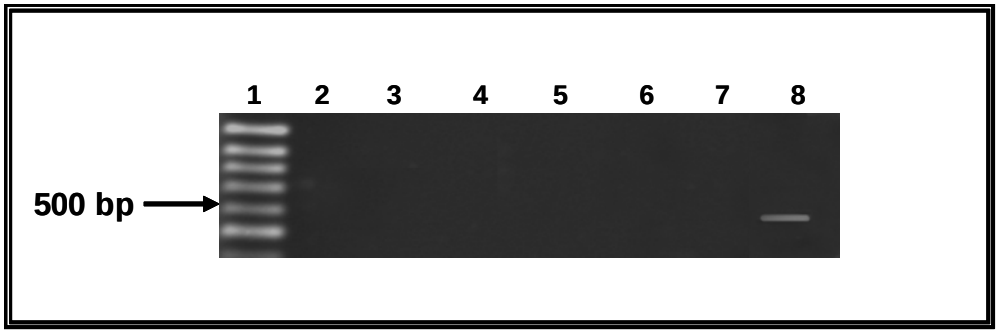

Fig. (31): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primer to *virA* gene. Lane (1) molecular marker DNA, lane (2, 3) transformed cv.capella plants, lane (4-6) transformed SWSR2 inbred line plants, lane (7) negative control (untreated plants) and lane (8) positive control (*Agrobacterium* DNA).

5.2.2- Using glass beads for wounding the tissue

a- Optimization of the wounding parameters

In the present experiments two different factors (speeds and durations of shaking with glass beads for wounding the tissue) were investigated at the same time.

Fig. 32A exhibited that the fluorometric GUS activity of cv.capella drastically decreased with increasing the speed and/or duration of the agitation. The higher speeds of agitation were highly effective on the fluorometric GUS activity than the lower speeds at 10-20 sec. However, when the explants were agitated for 10 sec. at 1000 rpm, the fluorometric GUS activity was 2.1 fold higher than the corresponding values at 2000 rpm. Interestingly, no GUS activity was noticed when the shaking was applied for 60 sec at 2000 rpm.

The histochemical GUS activity results of cv.capella proved the fluorometric results and achieved the highest *gus* expression frequency (28.6%) at 1000 rpm for 10 sec. Increasing the time of agitation to 20 sec. resulted in the reduction of *gus* expression 10.5 % in compare to the agitation for 10 sec. at 1000 rpm (Table 25).

The similar results were obtained from cell vitality which showed a dramatic reduction in the cell vitality as the speeds and/or duration increased. Explants were slightly affected by shaking at 1000 rpm for 10 sec (Fig. 32B)

As far as the regeneration frequency and efficiency is concerned, there was an inversely proportion between the regeneration properties of cv.capella and the speed and/or duration of the agitation process. Application of shaking for 10 sec. at 1000 rpm had a slight effect on the regeneration frequency as well as efficiency which recorded 55% and 4.0 regeneration score, respectively. The strongly effect of increasing the shaking speeds and/or duration appeared at 2000 rpm for 60 sec (Table 25).

With regard to Fig. 33A appeared a directly proportion between the fluorometric GUG activity of SWSR2 and the duration of agitation at 1000 rpm speed, while there were slight differences between the fluorometric values when the duration of agitation increased from 10 to 60 sec. using 2000 rpm speed of agitation. The highest fluorometric GUG activity was 366.9 µmol MU/mg protein /min. and resulted from agitation of the explants for 60 sec. at 1000 rpm speed.

In SWSR2 inbred line, the histochemical GUS assay did not clarify any *gus* expression in the plants when explants were agitated up to 20 sec. at 1000 rpm whereas, the frequency of *gus* expression recorded 30% with increasing the time of agitation to 60 sec. at the same speed. In addition, under the high speed of agitation (2000 rpm) the *gus* expression was decreased from 16.6 to 0.0% as the duration of agitation increased from 10 to 40 or 60 sec., respectively (Table 25).

In terms of the cell vitality of SWSR2 inbred line, there was a steadily reduction in the cell vitality with increasing the duration and/or the speed of agitation. The higher speed of agitation (2000 rpm) had a strongly bad effect on the cell vitality than the lower speed (1000 rpm) (Fig. 33B).

Wounding by glass beads caused a drastic reduction in the regeneration frequency and efficiency and was dependent on both speed and duration of agitation. This reduction in the regeneration properties was clearly noticed when the explants were submitted to beating at 2000 rpm. At lower speed (1000 rpm) the developing potential of the explants was largely dependent on the duration of the treatment. Exposure times >40 sec. greatly affected explant development (Table 25).

In addition, the most commonly observation was callus formation on the wounded explants. Amount of the induced callus was increased with increasing the speeds and/or durations of agitation.

Therefore, agitation of cv.capella explants at 1000 rpm for 10 sec. and SWSR2 explants at 1000 rpm for 60 sec. was applied in the following transformation experiments as a comprise between transformation efficiency (expressed as fluorometric GUS activity) and cell vitality

b- Estimation of transformation frequency

In this transformation experiment, split shoot apices of cv.capella were agitated with *Agrobacterium* suspension for 10 sec. at 1000 rpm, while the agitation of SWSR2 inbred line explants was for 60 sec. at the same speed. However, plants were subjected to histochemical, fluorometric and molecular analysis and the obtained results were presented in Table 26 and Fig. 34 and 35.

❖ ***Histochemical GUS assay***

Gus expression was detected only in two and one plant of cv.capella and SWSR2 inbred line, respectively, whereas, no *gus* expression was observed in untreated plants (control) (Table 26).

❖ ***Fluorometric GUS assay***

The fluorometric results showed that the hybrid had a superior response than the inbred line and recorded 823.3 µmol MU/mg protein/min. Meanwhile, the fluorometric GUS activity of the inbred line was 376.9 µmol MU/mg protein/min. (Table 26).

❖ ***PCR analysis of transformants***

The transformed plants of both genotypes were positive for PCR analysis using *gus* or *nptII* primers, whereas the non-transformed tissues showed negative results.

Two and one positive plants of cv.capella and SWSR2 inbred line, respectively, showed amplified band at 830 and 804 bp with *gus* and *nptII* primers, respectively (Fig. 34). The transformation frequency was 1.7% and 0.9% for cv.capella and SWSR2, respectively. This frequency was calculated on the basis of positive PCR

plants. No amplified band was detected in the positive plants using *virA* primer in both genotypes (Fig. 35).

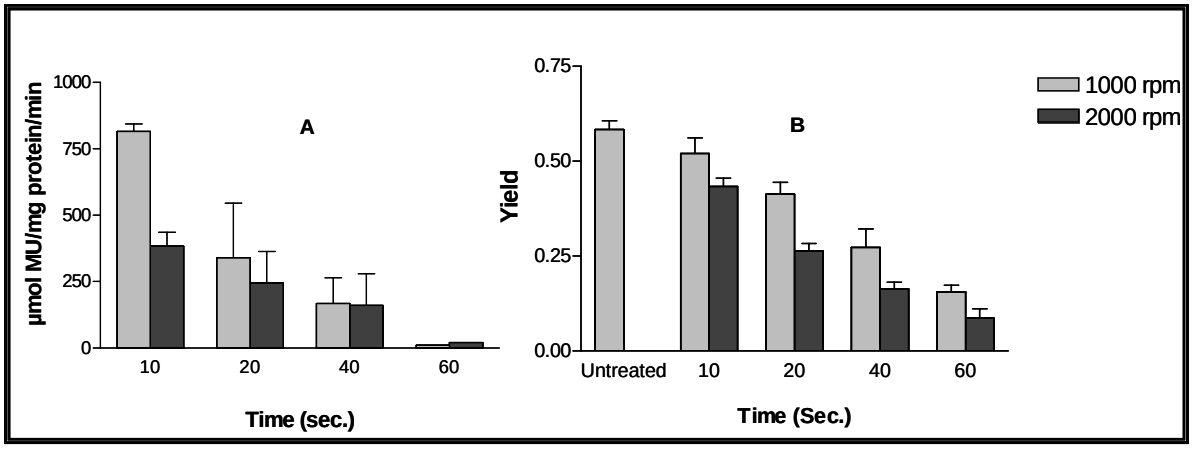

Fig. (32): Evaluation of different glass wounding parameters coupled with *Agrobacterium* infiltration method of split shoot apices for high oleic *H. annuus* L. cv.capella using fluorometric GUS activity and the cell vitality. A: fluorometric GUS activity and B: cell vitality. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer) and results are means of four replicates ± SE.

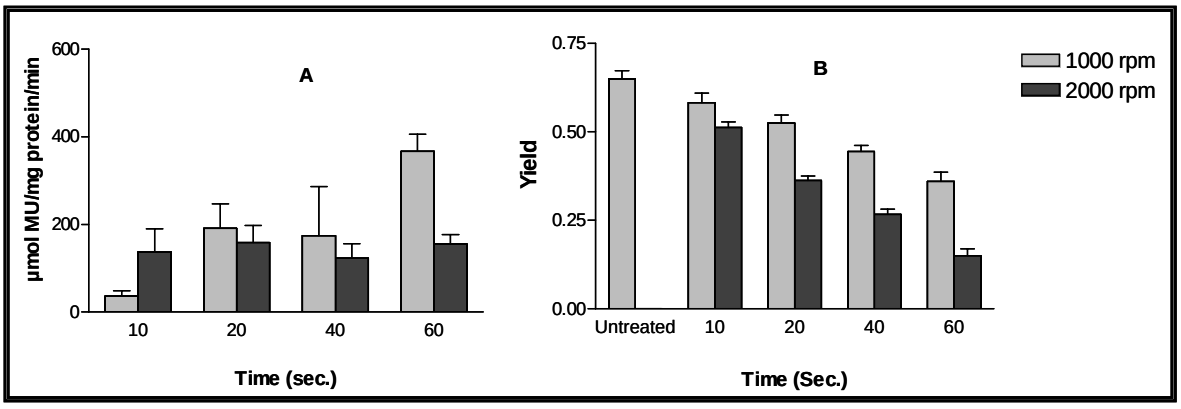

Fig. (33): Assessment of different glass wounding parameters coupled with *Agrobacterium* infiltration method of split shoot apices for high oleic *H. annuus* L. SWSR2 inbred line using fluorometric GUS activity and the cell vitality. A: fluorometric GUS activity and B: cell vitality. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer) and results are means of four replicates ± SE.

Table (25): Effect of different glass wounding parameters, wounding speeds and durations, on the regeneration percentage and efficiency and histochemical GUS assay of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Wounding parameters	Regeneration (%) Mean ± SE		Regeneration efficiency* Mean ± SE		Callus induction		GUS expressing shoots (%)	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
Untreated	62.5 ± 2.5	65.0 ± 2.8	4.7 ± 0.06	4.5 ± 0.10	+	++	0.0	0.0
1000 rpm								
10 sec.	55.0 ± 2.8	57.5 ± 2.5	4.0 ± 0.12	4.0 ± 0.15	++	++	28.6	0.0
20 sec.	47.5 ± 4.7	52.5 ± 4.7	3.6 ± 0.11	3.8 ± 0.08	++	++	18.1	0.0
40 sec.	30.0 ± 4.0	47.5 ± 2.5	3.0 ± 0.20	3.4 ± 0.10	+++	+++	14.3	15.4
60 sec.	15.0 ± 2.8	37.5 ± 4.7	2.0 ± 0.28	2.9 ± 0.22	+++	+++	0.0	30.0
2000 rpm								
10 sec.	47.5 ± 2.5	45.0 ± 5.0	3.1 ± 0.14	3.6 ± 0.14	++++	+++	14.3	16.6
20 sec.	35.0 ± 2.8	35.0 ± 2.8	2.7 ± 0.10	2.9 ± 0.23	++++	++++	11.1	10.0
40 sec.	15.0 ± 2.8	22.5 ± 4.7	1.6 ± 0.13	2.0 ± 0.11	++++	++++	0.0	0.0
60 sec.	2.5 ± 2.5	7.5 ± 2.5	1.0 ± 0.09	1.2 ± 0.12	++++	++++	0.0	0.0

(*) Regeneration efficiency was expressed as a score from 1 to 5 and means are values of four replicates. Callus induction score was + = no callus induction, ++ = little callus induction, +++ = moderate callus induction and ++++ = abundant callus induction.

Table (26): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using glass wounding system combined with *Agrobacterium* infiltration method.

Genotype	Total No of co-cultivated explants	No of + ve Gus plants	Gus frequency* (%)	Fluorometric assay (µmol MU/mg protein/min.) Mean + SE	No of tested PCR plants	No of + ve PCR plants	Transformation frequency* (%)
cv.capella	112	2	1.7	823.3 ± 28.2	30	2	1.7
SWSR2	110	1	0.9	376.9 ± 39.2	30	1	0.9

(*) The percentage was calculated in relation to the total number of co-cultivated explants and PCR was performed with *gus* and *nptII* primers 14-16 weeks after co-cultivation. Transformation frequency was calculated on the basis of positive PCR plants.

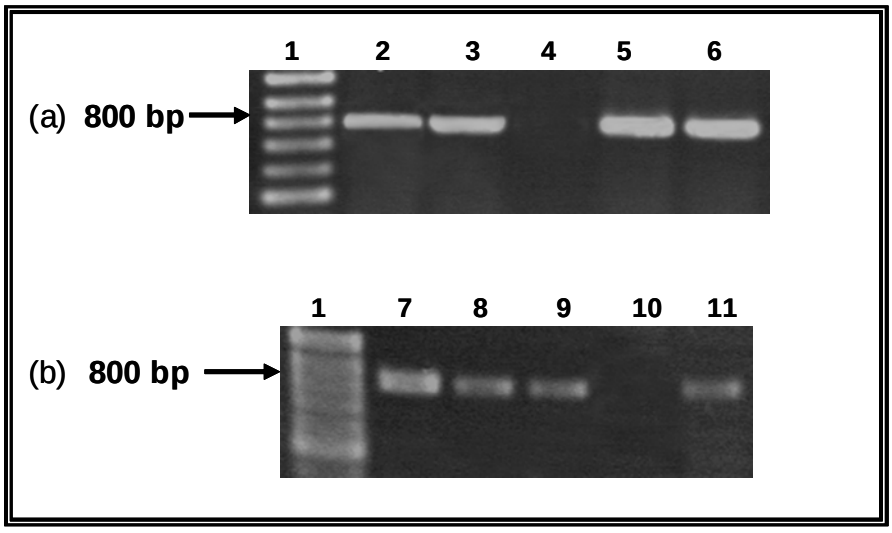

Fig. (34): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2, 3 and 7, 8) transformed cv.capella plants, lane (4, 10) negative control (untreated plants), lane (5, 11) positive control (pure plasmid) and lane (6 and 9 and) transformed SWSR2 inbred line plants.

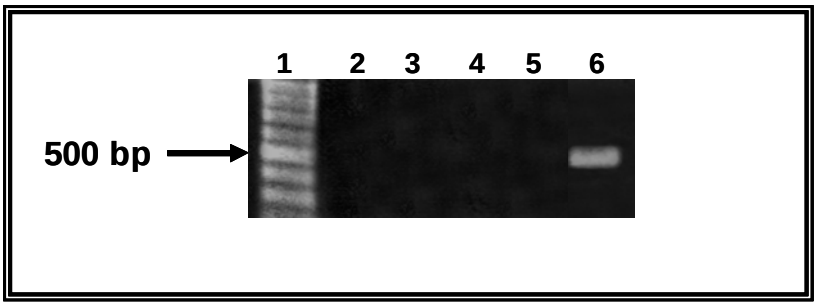

Fig. (35): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primer to *virA* gene. Lane (1) molecular marker DNA, lane (2, 3) transformed cv.capella plants, lane (4) transformed SWSR2 inbred line plants, lane (5) negative control (untreated plants) and lane (6) positive control (*Agrobacterium* DNA).

5.3- *Agrobacterium* injection

5.3.1-Assessment of injection parameter

In this investigation two sizes of injection capillary were used in an effort to reach and inject the meristematic cells with *Agrobacterium* suspension hence, increasing the transformation incidence of cv.capella and SWSR2 inbred line. Effect of different injection capillary size on the fluorometric and histochemical GUS activity as well as the cell vitality was demonstrated in Fig. 36 and Table 27.

As shown in Fig. 36 injection of cv.capella explants with *Agrobacterium* suspension OD_{600} = 1 throw 7-9 µm injection capillary size resulted in 196.8 µmol MU/mg protein /min., while no fluorometric GUS activity was recorded from SWSR2 inbred line using the same size of the capillary. When the wide capillaries (25-27µm) were used for injection, no fluorometric results were obtained from both genotypes.

Table 27 showed also the similar results from the histochemical GUS assay which proved that injection of the explants with the narrow capillary (7-9 µm) resulted in 10% of the plants expressing GUS activity. Meanwhile no *gus* expression was detected from the other treatment.

As far as the regeneration properties are concerned, there was a slight effect on the regeneration efficiency using 7-9 µm capillary size. On the other hand, the regeneration

frequency decreased from 60.0 to 46.6% and from 63.3 to 50.0% with increasing the capillary size from 7-9 to 25-27 µm in cv.capella and SWSR2 inbred line, respectively.

These results were also confirmed with cell vitality results which gave the highest cell vitality values (0.52 and 0.51 yield) of cv.capella and SWSR2 inbred line, respectively, by using 7-9 µm capillary size comparing with the other treatment.

5.3.2- Estimation of transformation frequency

Injected plants with *Agrobacterium* suspension from this transformation experiment were subjected to histochemical, fluorometric and molecular analysis and the obtained results were presented in Table 28 and Fig. 37, 38.

❖ ***Histochemical GUS assay***

No histochemical GUS activity was observed in both transformed and non-transformed tissues of both genotypes (Table 28).

❖ ***Fluorometric GUS assay***

Similarly, no fluorometric GUS activity results were recorded from SWSR2 inbred line, whereas cv.capella had achieved 204.8 µmol MU/mg protein/min. from this assay (Table 28).

❖ ***PCR analysis of transformants***

Despite of the negative results of the histochemical assay of both genotypes, one plant of cv.capella showed amplified band with either of the two primers (*gus* and *nptII*) (Fig. 37). Moreover, an additional PCR reaction was performed for the transformed and non-transformed (control) samples with *virA* primer as an evidence for the absence of *Agrobacterium* contamination, only in the positive control 500 bp band was amplified (Fig. 38)

Transformation frequency of *Agrobacterium* injection method was estimated on the basis of positive PCR plants and in relation to the total number of co-cultivated explants. Cv.capella had achieved 3.3% transformation frequency and on the other hand, no transformation event was detected in SWSR2 inbred line plants.

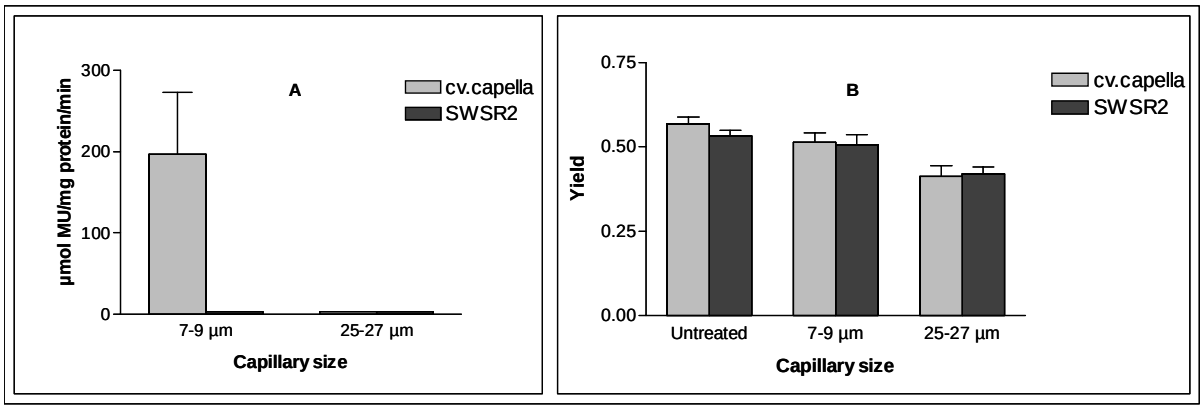

Fig. (36): Assessment of different injection capillary sizes for *Agrobacterium* injection of split shoot apices for two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line using fluorometric GUS activity and the cell vitality. (A): fluorometric GUS activity and (B) Cell vitality. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer) and results are means of at least four replicates ± SE.

Table (27): Effect of different injection capillary sizes for *Agrobacterium* injection on the regeneration percentage and efficiency and histochemical GUS assay of split shoot apices of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

Injection parameter	Regeneration % Mean ± SE		Regeneration efficiency* Mean ± SE		Gus expressing shoots %	
	cv.capella	SWSR2	cv.capella	SWSR2	cv.capella	SWSR2
Capillary size						
Untreated	63.3 ± 3.3	66.6 ± 3.3	4.6 ± 0.18	4.4 ± 0.08	0.0	0.0
7-9 µm	60.0 ± 5.7	63.3 ± 6.6	4.3 ± 0.12	4.0 ± 0.10	10.0	0.0
25-27 µm	46.6 ± 3.3	50.0 ± 5.7	3.2 ± 0.20	3.4 ± 0.24	0.0	0.0

(*) Regeneration efficiency was expressed as a score from 1 to 5 and means are values of three replicates.

Table (28): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using *Agrobacterium* injection method.

Genotype	Total No of co-cultivated explants	No of + ve Gus plants	Gus frequency* (%)	Fluorometric assay (µmol MU/mg protein/min.) Mean + SE	No of tested PCR plants	No of + ve PCR plants	Transformation frequency* (%)
cv.capella	30	0	0	204.8 ± 75.9	15	1	3.3
SWSR2	30	0	0	0.0 ± 0.0	17	0	0.0

(*) The percentage was calculated in relation to the total number of co-cultivated explants and PCR was performed with *gus* and *nptII* primers after 14-16 weeks of co-cultivation. Transformation frequency was calculated on the basis of positive PCR plants.

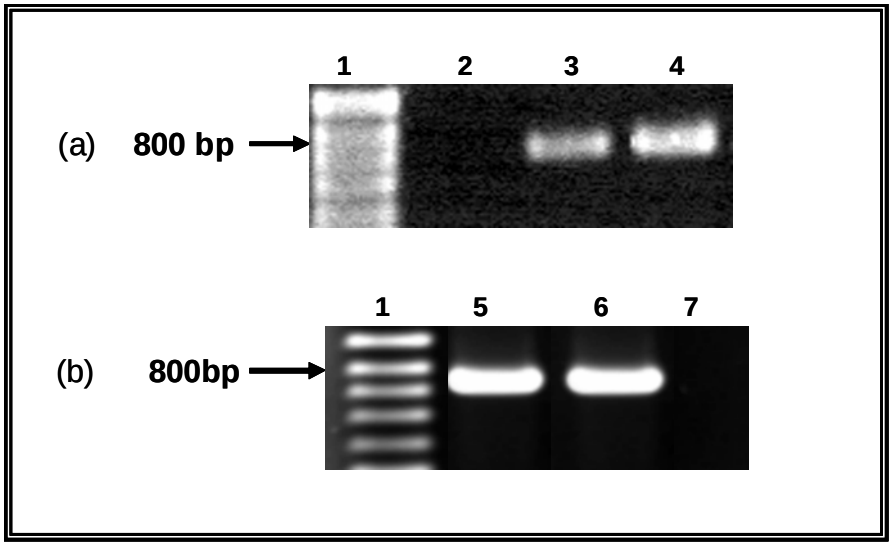

Fig. (37): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2, 7) negative control (untreated plants), lane (3, 5) transformed cv.capella plants and lane (4, 6) positive control (pure plasmid).

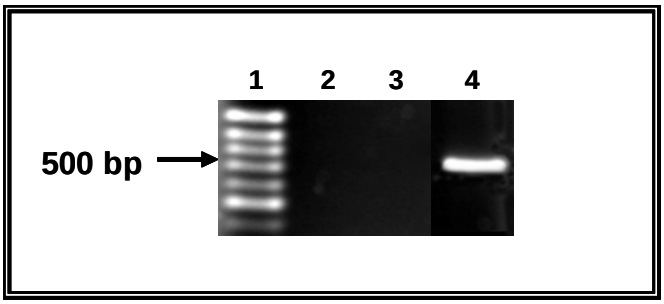

Fig. (38): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primer to *virA* gene. Lane (1) molecular marker DNA, lane (2) transformed cv.capella plants, lane (3) negative control (untreated plants) and lane (4) positive control (*Agrobacterium* DNA).

5-4- Biolistic gene transfer

5-4.1- Evaluation of different physical and biological parameters

A set of various physical and biological parameters, different microcarrier gold particles sizes (1 and 1.6 µm), particle acceleration pressures (0, 450, 900, 1550 and 1800 psi), distances between macrocarrier assembly and target plate (6 and 9 cm), pre-culture durations of the explant (0, 1 and 2 days) and number of bombardments / explant (1 and 2 shot), that support the introduction of DNA with minimal tissue damage or interference with the regeneration potential, were optimized in a single or multifactorial way using pBI121 coated gold particles for cv.capella hybrid and SWSR2 inbred line. Fig. 39 and 40 presented the effect of gold particle size, particle acceleration pressure (helium pressure) and target distance on the fluorometric, histochemical, cell vitality as well as shoot induction frequency of cv.capella and SWSR2, respectively.

The general pattern of response was similar in both tested genotypes with respect to the recorded values. The highest fluorometric values were always resulted from using 1.6 µm particles size with regardless of the helium pressure and target distance used. Moreover, There was a directly proportion between the fluorometric GUS activity and the helium pressure up to 1550 psi, then increasing the helium pressure decreased the fluorometric activity.

Increasing the target distance to 9 cm induced a reduction in the fluorometric GUS activity with respect to the particles size and the helium pressure used. In comparison, the fluorometric GUS activity of cv.capella and SWSR2 inbred line plants which were bombarded with 1.6 µm particles size and 1550 psi at 9 cm was 3.7 and 2.9 times lower than those bombarded with the same particles size and helium pressure at 6 cm, respectively (Fig. 39A and 40A). Increasing the target distance to 9 cm could not be compensated by the elevation of acceleration pressure with the use of any particles size.

As far as the *gus* expression frequency is concerned, a helium pressure of 1550 psi in combination with a target distance 6 cm and 1.6 µm particles size resulted in the highest *gus* expression frequency which amounted to 33.3 and 30.8 % for cv.capella and SWSR2 inbred line, respectively. There was no *gus* expression observed in the plants which bombarded at 9 cm and 450-900 psi using either particles size of both genotypes (Fig. 39B and 40B).

With regard to Fig. 39C and 40C, appeared that the cell vitality of the bombarded tissues was inversely related to the helium pressure at either of the two target distances. At the highest helium pressure (1800 psi) the bombarded tissues with 1.6 µm particles size at 6 cm distance were extensively damaged in both genotypes.

Overall, shoot induction frequency was indeed influenced by varying helium pressure. This influence was more obvious at 6 cm target distance with 1.6 µm particles size. Using a combination of 1.6 µm particles size, 6 cm target distance and 1800 psi decreased the shoot induction frequency to 12.5 and 7.5 % of cv.capella and SWSR2 inbred line, respectively (Fig. 39D and 40D).

Considering the overall effects of acceleration pressure on the fluorometric and histochemical GUS activity as well as the cell vitality and the tissue culture response, a helium pressure of 1550 psi, combined with 6 cm target distance and 1.6 µm particles size was found to be acceptable for bombardment of sunflower split shoot apices.

In a series of experiments, all the optimized parameters (shown above) were applied in subsequent transformation experiments. These experiments were designed to find out the effect of pre-culture duration and number of shots per explant on the transformation events as well as cell vitality and tissue culture response.

As presented in Fig. 39E and 40E that bombardment the explants two times resulted in the highest levels of fluorometric GUS activity. In comparison, when the

explants were bombarded twice after one day of pre-culture, an increase in fluorometric GUS activity of 1.6 and 2.1 fold was achieved in cv.capella and SWSR2 inbred line, respectively, compared to explants bombarded once.

Moreover, pre-culture duration also affected fluorometric GUS activity, when explants were cultured for one day prior to bombardment, higher fluorometric values were obtained compared to 0 and 2 days of culture.

In terms of histochemical assay, the *gus* expression decreased 13 and 17.5% with increasing the pre-culture duration to 2 days in cv.capella and SWSR2 inbred line, respectively, when explants were bombarded once. Meanwhile, there was a variation in the general pattern of *gus* expression between the two genotypes when the explants were bombarded twice. The lowest *gus* expression frequency was 12.5% in cv.capella and was resulted from pre-culture the explants for one day prior to bombardment twice. Conversely, the highest *gus* expression frequency was 25% in SWSR2 inbred line and resulted from the same condition (Fig. 39E and 40E).

Influence of pre-culture duration and number of shots per explant on the shoot induction and the cell vitality was illustrated in Fig. 39F and 40F. Shoot induction frequency and the cell vitality increased as the pre-culture duration increased. Number of bombardment per explant affected shoot induction frequency as well as the cell vitality. It was clear that cv.capella was deeply affected with increasing the number of shots per explants compared to SWSR2 inbred line.

In conclusion, on the basis of the results obtained during the optimization experiments, the adapted bombardment conditions are: 1550 psi of acceleration pressure in combination with 6 cm target distance, 1.6 µm gold particle size and pre-culture the explants for one day prior to bombardment twice.

5-4.2- Estimation of transformation frequency

On the basis of optimized biolistic parameters (mentioned above), transformation experiments were performed using split shoot apices of cv.capella and SWSR2 inbred line. Plants were subjected to histochemical, fluorometric and molecular analysis and the transformation results were presented in Table 29 and Fig. 41, 42.

❖ ***Histochemical GUS assay***

As shown in Table 29 the recorded number of *gus* expressing plants was 1 and 4 in cv.capella and SWSR2 inbred line, respectively. *gus* expression was completely and uniformly in the transformed plants, while no expression was detected in the untreated plants (control) (see Fig. 44).

❖ ***Fluorometric GUS assay***

The fluorometric GUS activity results reflected that SWSR2 inbred line had a higher response than the cv.capella for the transformation using the present method. The fluorometric value of SWSR2 was 1723.2 µmol MU/mg protein/min. whereas, the corresponding value recorded from cv.capella was 1509.6 µmol MU/mg protein/min. (Table 29).

❖ ***PCR analysis of transformants***

The specific amplified fragments of 830 and 804 bp for *gus* and *nptII*, respectively, could be amplified in the transformed plants of cv.capella and SWSR2 inbred line (Fig. 41), whereas no amplified band was detected in the non-transformed plants. Out of 30 and 40 tested PCR plants only 3 and 5 plants were positive with either of the two primers of cv.capella and SWSR2 inbred line, respectively (Table 29). Interestingly, some of the PCR positive plants did not express the *gus* gene. The transformation frequency of cv.capella was 3.1%, while the corresponding frequency of SWSR2 was 4.5% in relation to the total number used in the experiment.

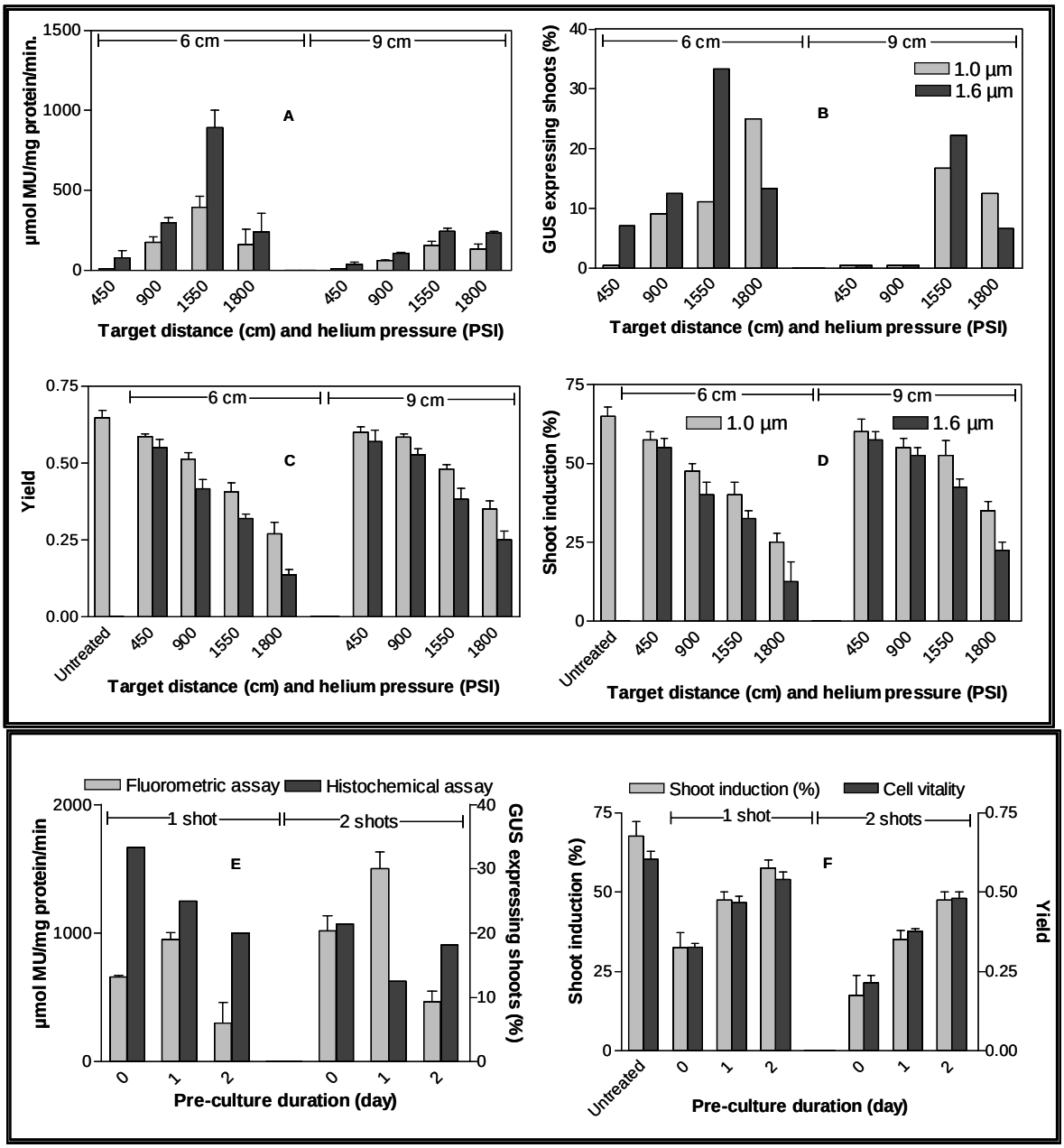

Fig. (39): Assessment of different particle bombardment parameters enhancing the transformation efficiency of split shoot apices for high oleic *H. annuus* L. cv.capella on the basis of fluorometric and histochemical GUS activity coupled with shoot induction percentage and the cell vitality. A, B, C and D: different target distances, different helium pressures and different gold particle sizes, the bombardment were applied without pre-culture the explants and single shot per plate, E and F different pre-culture durations and different number of shots per plate, where not specified, bombardment were applied under 6 cm distance between macrocarrier assembly and target plate using 1.6 µm gold particles and 1550 (PSI) helium pressure. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer). Results are data of at least three replicates and errorbars represented SE.

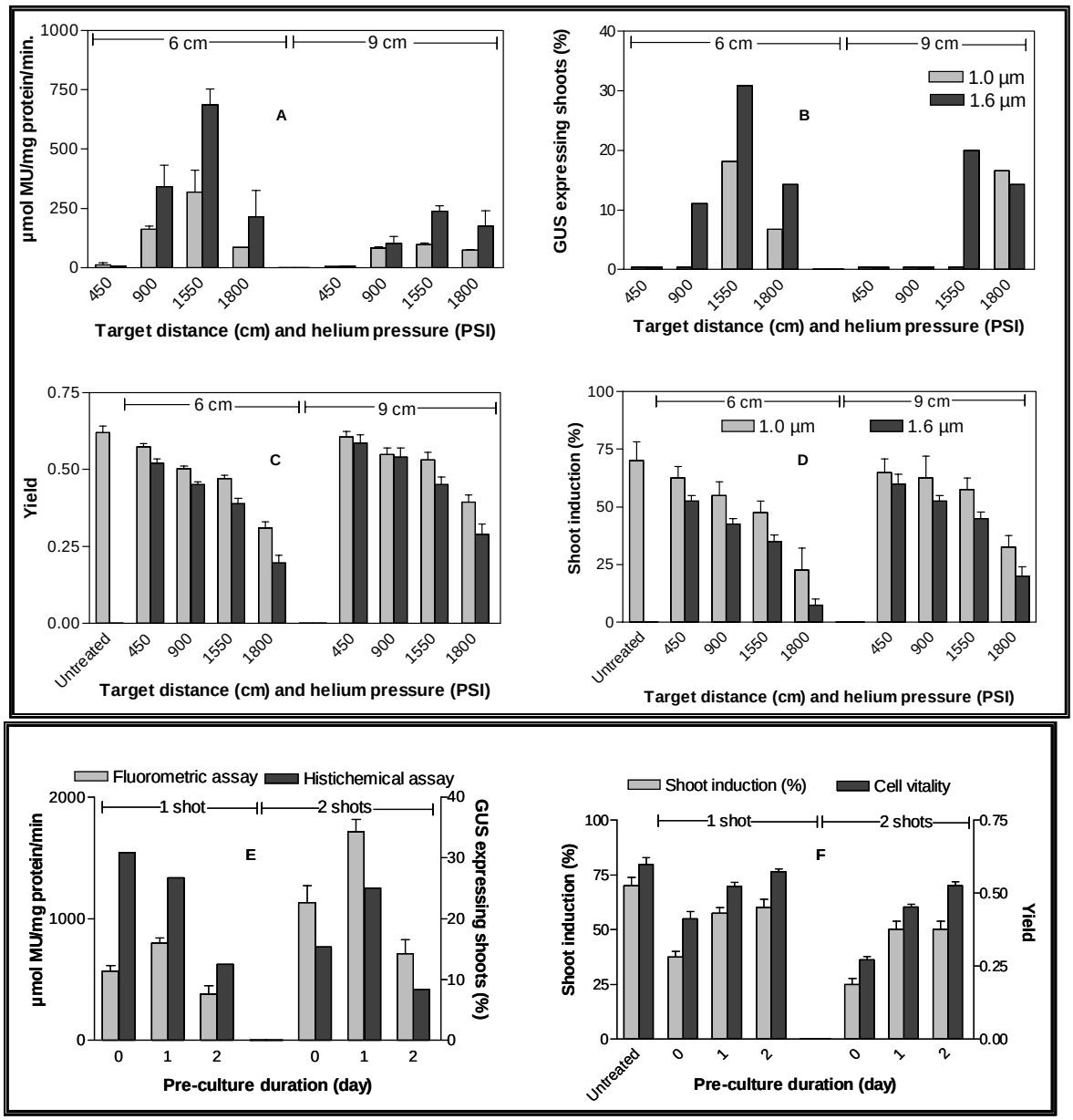

Fig. (40): Assessment of different particle bombardment parameters enhancing the transformation efficiency of split shoot apices for high oleic *H. annuus* L. SWSR2 inbred line on the basis of fluorometric and histochemical GUS activity coupled with shoot induction percentage and the cell vitality. A, B, C and D: different target distances, different helium pressures and different gold particle sizes, the bombardment were applied without pre-culture the explants and single shot per plate, E and F different pre-culture durations and different number of shots per plate, where not specified, bombardment were applied under 6 cm distance between macrocarrier assembly and target plate using 1.6 µm gold particles and 1550 (PSI) helium pressure. The vitality was expressed as a mean of the yield (PAM 2000 fluorometer). Results are data of at least three replicates and errorbars represented SE.

Table (29): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using biolistic gene transfer method.

Genotype	Total No. of used explants	No of + ve Gus plants	Gus frequency* (%)	Fluorometric assay (µmol MU/mg protein/min.) Mean + SE	No of tested PCR plants	No of + ve PCR plants	Transformation frequency* (%)
cv.capella	95	1	1.1	1509.6±130.8	30	3	3.1
SWSR2	110	4	3.6	1723.2±101.3	40	5	4.5

(*) The percentage was calculated in relation to the total number of bombarded explants and PCR was performed with *gus* and *nptII* primers 14-16 weeks after bombardment. Transformation frequency was calculated on the basis of positive PCR plants.

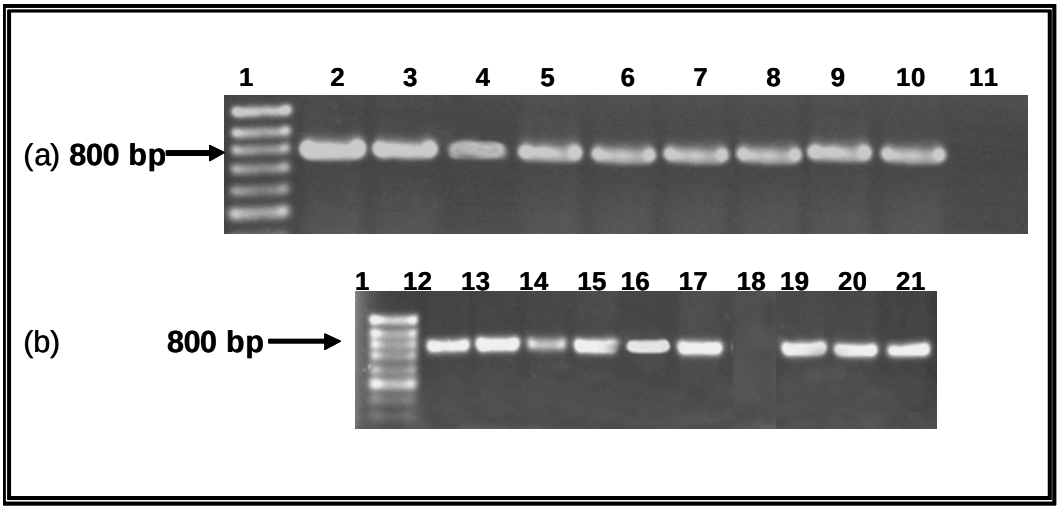

Fig. (41): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2-6 and 12-16) transformed SWSR2 inbred line plants, lane (7-9 and 19-21) transformed cv.capella plants, lane (10, 17) positive control (pure plasmid) and lane (11, 18) negative control (untreated plants).

In summary, Fig. 42 and 43 illustrated a summary of all the transformation experiments which carried out to select the most efficient method for the two high oleic *H. annuus* L. genotypes used in this study. This selection was based on the fluorometric GUS activity combined with shoot induction frequency.

Data in Fig. 42A indicate that *Agrobacterium* infiltration method has found to be a compromise between fluorometric GUS activity and shoot induction frequency for cv.capella. Using this method has achieved the highest fluorometric GUS activity (1953.6 µmol MU/mg protein /min.) and at the same time high frequency of shoot induction (60%). For SWSR2 biolistic gene transfer was selected as the best transformation method since recorded the highest fluorometric value (1723.2 µmol MU/mg protein /min.) and 50% shoot induction frequency (Fig. 42B).

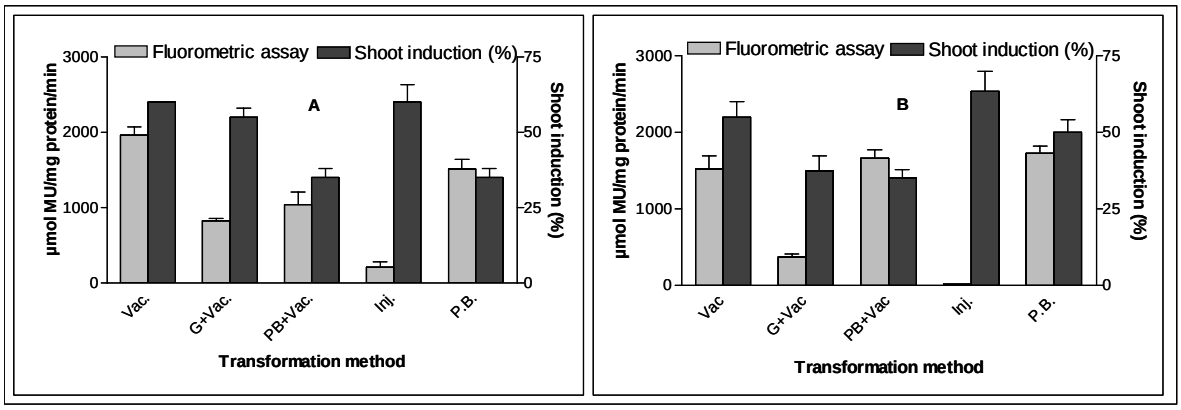

Fig. (42): Summary of different transformation methods experiments of split shoot apices from high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line on the base of fluorometric GUS activity combined with shoot induction frequency. Data are means of at least four replicates ± SE. (A) cv.capella and (B) SWSR2. Vac.= vacuum infiltration method, G+Vac.= glass wounding+vacuum infiltration, PB+Vac.= wounding using particle bombardment + vacuum infiltration, Inj= *Agrobacterium* injection and P.B.= particle bombardment (Biolistic gene transfer).

Eventually, these data were confirmed with results of the transformation frequency which calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated explants (Fig. 43). *Agrobacterium* infiltration method has recorded 4% transformation frequency for cv.capella, while the corresponding frequency of SWSR2 inbred line using biolistic gene transfer method

was 4.5%. Additionally, there was a variation in the transformation response between the two tested genotypes. In comparison, the transformation frequency of cv.capella was higher than the SWSR2 inbred line in the most transformation experiments. This indicates a pronounced effect of the genotype on the transformation frequency.

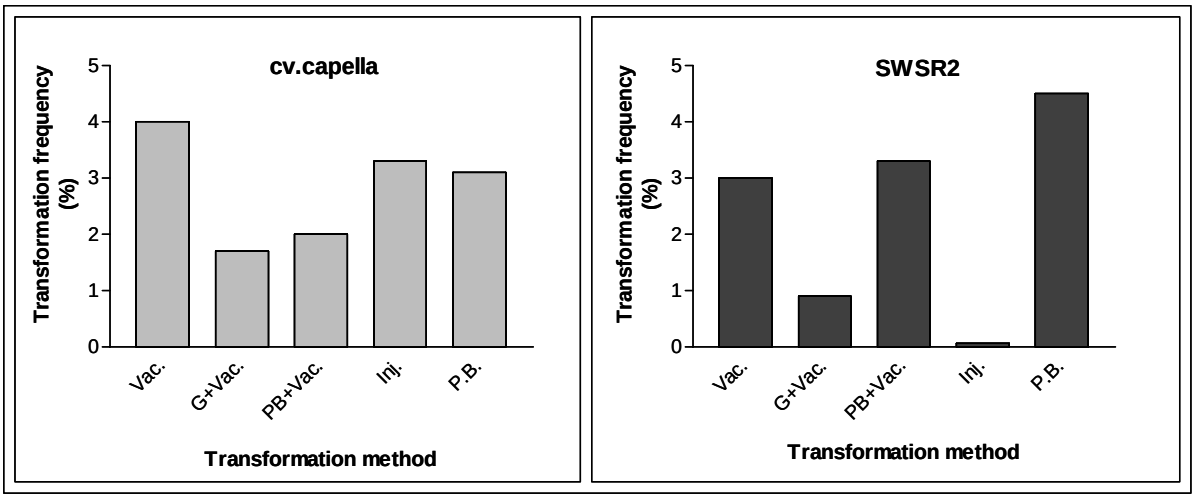

Fig. (43): Transformation frequency of split shoot apices from high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line using different transformation methods. The transformation frequency was calculated on the basis of PCR analysis after 14-16 weeks of co-cultivation and recorded as a percentage from the total number of co-cultivated explants. Vac.= vacuum infiltration method, G+Vac.= glass wounding+vacuum infiltration, PB+Vac.= wounding using particle bombardment + vacuum infiltration, Inj= *Agrobacterium* injection and P.B.= particle bombardment (Biolistic gene transfer).

6- Stable transformation

In these experiments the optimized transformation method for cv.capella and SWSR2 inbred line was applied on 120 and 125 split shoot apices, respectively, to test the reproducibility of the method and the stability of the transmitted gene in the next generation. For this purpose the T0 and T1 plants were subjected to histochemical, fluorometric and molecular analysis.

❖ *Histochemical GUS assay*

Gus expression was uniformly observed as a dark blue color in the transformed tissues (Fig. 44). On the other hand, non-transformed tissue did not present any blue

color. However, the GUS frequency amounted to 4.1 and 3.2% for cv.capella and SWSR2 inbred line, respectively (Table 30).

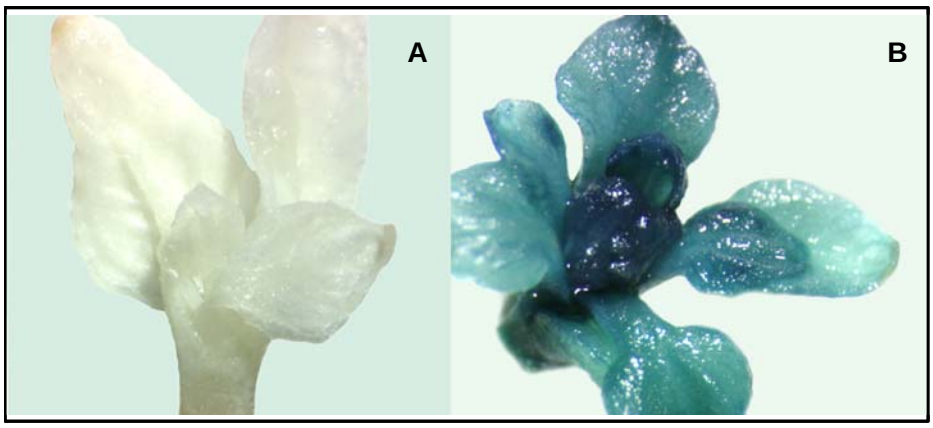

Fig. (44): *Gus* expression of the stable transformation experiment of high oleic sunflower genotypes, e.g. cv.capella. (A) *gus* expression in non transformed shoot and (B) GUS expressing shoot after GUS staining.

❖ ***Fluorometric GUS assay***

The initial screening was done by the fluorometric assay 5 weeks after co-cultivation for all regenerants. Five and six regenerants of cv.capella and SWSR2 inbred line, respectively, showed high fluorometric values and those have been tested 10 and 12-14 weeks after co-cultivation. There was a detectable variation in the *gus* expression into the same plant during a period of 12-14 weeks. The GUS activity of plant number 2 and 4 of cv.capella and number 3 and 6 of SWSR2 remained almost stable during all stages of plant development over 12-14 weeks whereas, plant number 1 and 3 of cv.capella and plant number 4 and 5 of SWSR2 appeared a gradual reduction in the GUS activity during the same duration (Fig. 45) . Another pattern of variation in the *gus* expression was noticed in plant number 5 of cv.capella and number 1 of SWSR2. In these plants, the *gus* expression decreased from 1740 to 1330 and from 1730.3 to 1100 µmol MU/mg protein /min., respectively, compared to those after 5 weeks, while there was no reduction was detected after 12-14 weeks of both plants. Moreover, plant number 2 of SWSR2 presented stability in the *gus* expression over 10 weeks followed by about 19% reduction in the GUS activity after 12-14 weeks (Fig. 45B).

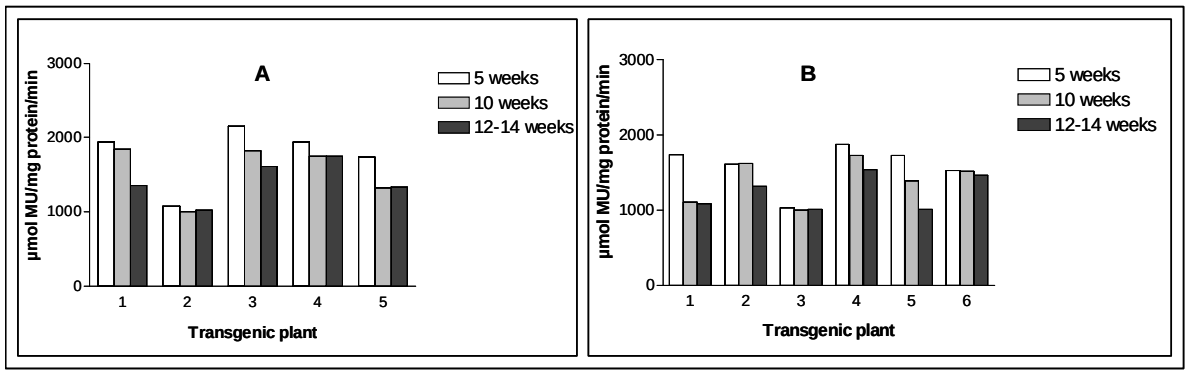

Fig. (45): Variation of fluorometric GUS activity among T_0 plants over a period of 12-14 weeks on different development media, one leaf was tested each time. (A) cv.capella and (B) SWSR2.

❖ *PCR analysis of transformants*

PCR analysis was carried out as a rapid identification for the presence of T-DNA in the positive fluorometric plants in addition to some random samples. Fig. 46a clarified the amplification of 830 bp fragments corresponding to *gus* gene. Meanwhile, no amplification was detected in the samples from non-transformed plants. Moreover, an additional PCR reaction was performed using primer specific for *nptII* gene. The appearance of the amplified band at 804 bp confirmed the transmission of the selectable marker gene to the transgenic plants (Fig. 46b). Interestingly, some of the PCR positive plants could not express the *gus* gene. The transformation frequency of cv.capella amounted to 4.1% using *Agrobacterium* infiltration method whereas, it was 4.8% in SWSR2 inbred line using direct gene transfer method. This frequency was calculated on the basis of positive PCR plants and recorded as a percentage from the total number of co-cultivated or bombarded explants (Table 30).

Table (30): Summary of stable transformation events of T_0 plants of high oleic *H. annuus* L. genotypes, cv.capella using *Agrobacterium* infiltration and SWSR2 using biolistic gene transfer method.

Genotype	Total No. of used explants	No of +ve Gus plants	Gus frequency* (%)	No of tested PCR plants	No of +ve PCR plants	Transformation frequency* (%)
cv.capella	120	5	4.1	40	5	4.1
SWSR2	125	4	3.2	40	6	4.8

(*) The percentage was calculated in relation to the total number of used explants and PCR was performed with *gus* and *nptII* primers 14-16 weeks after co-cultivation or bombardment. Transformation frequency was calculated on the basis of positive PCR plants.

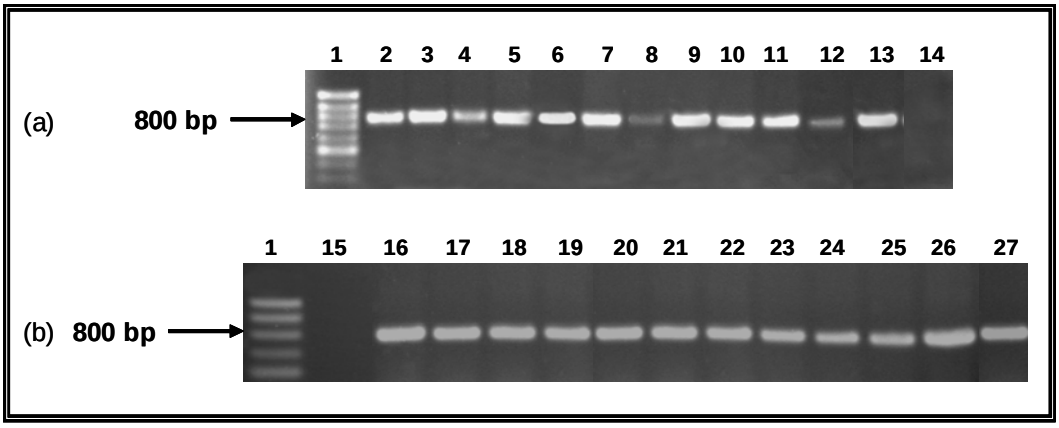

Fig. (46): PCR analysis of T_0 plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primer and (b) with *nptII* primer, lane (1) molecular marker DNA, lane (2-6 and 17-21) transformed cv.capella plants, lane (7-12 and 22-27) transformed SWSR2 inbred line plants, lane (13,16) positive control (pure plasmid) and lane (14,15) negative control (untreated plants).

❖ ***Southern blot analysis of T_0 plants***

Southern blot analysis was conducted and labelling of DNA was first attempted by enhanced chemiluminescence (ECL Kit), but no results were obtained. Consequently, the sensitivity of the method was tested using different concentrations of pure plasmid pBI121. The dot blot experiment clarified that this method is not sensitive to < 640 ng DNA (data not shown). Accordingly, it was found that this method was not appropriate for the southern blots with genomic DNA. Labelling the DNA with [α-^{32} P] –dCTP using the "Random primed hexalabelling DNA Kit" revealed a positive results.

Southern blot analysis of some independent T_0 plants confirmed the presence and integration of the *gus* fragment gene into the sunflower genome of both genotypes (Fig. 47).

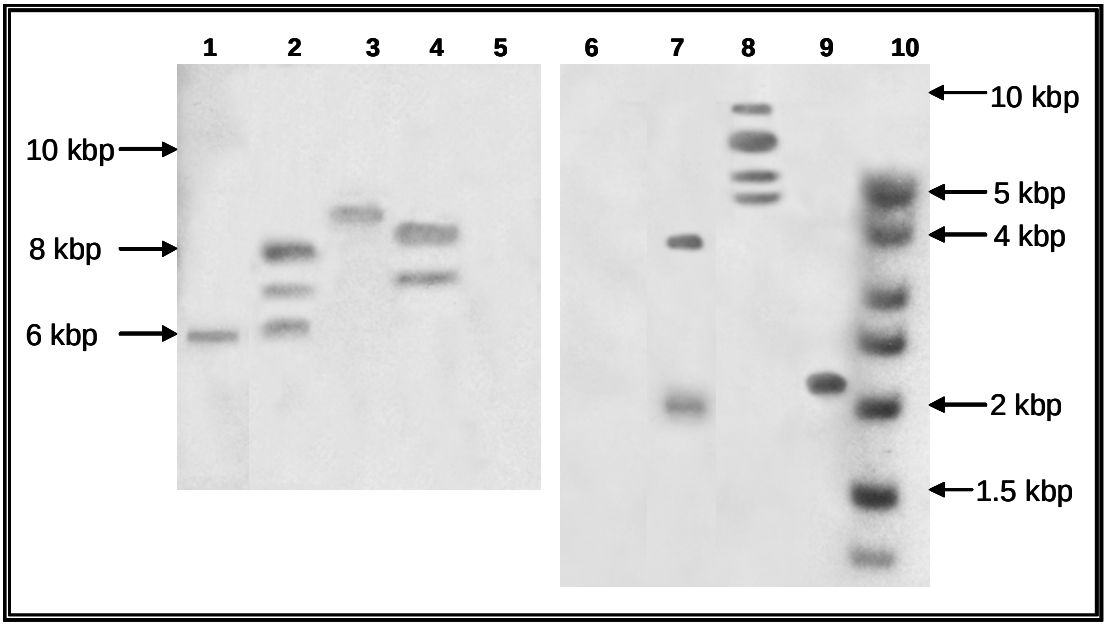

Fig. (47): Southern blot hybridization of random independent T_0 transgenic plants of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line. Genomic DNA was digested with *Eco*RI and hybridised with radio labelled *gus* probe. Lane 1, 3, 7, 9 transgenic cv.capella plants, lane 2, 4, 8 transgenic SWSR2 inbred line plants, lane 5, 6 non-transformed sunflower and lane 10 marker DNA.

Eventually, single or two hybridising bands were observed in cv.capella plants which transformed with *Agrobacterium* infiltration method (lane 1, 3, 7 and 9).

Meanwhile, multiple insertion events of two to four copies of *gus* gene into SWSR2 inbred line genome were detected in the transformed plants using biolistic gene transfer (lane 2, 4 and 8). This proves the stable integration of the *gus* gene into the two high oleic *H. annuus* L. genotypes. Conversely, no hybridising band was observed in non-transformed cv.capella or SWSR2 inbred line (lane 5 and 6)

❖ ***Transgene expression and inheritance in the T_1 progenies***

The *gus* gene was stably transmitted and expressed in subsequent sexual generation of most of the analysed plants of both genotypes (Table 31 and 32). Plant number 5 of cv.capella and plants number 2, 6 of SWSR2 inbred line showed non-detectable expression levels in subsequent generation either in histochemical assay or PCR analysis. In SWSR2 inbred line, although the progeny of plant number 5 did not show any *gus* expression, three of them were positive in PCR analysis (Table 32).

As far as southern blot analysis is concerned, T_1 plants from random T_0 plants of both genotypes were analysed. Four insertion events with two copies of *gus* gene were observed in the progeny of transgenic cv.capella (Fig. 48 A lane 1-4) The transformed plants by biolistic gene transfer had three copies of *gus* gene (Fig. 48 B lane 1, 3, 4). Conversely, no hybridising band was observed in non-transformed cv.capella or SWSR2 inbred line (lane 5 and 2).

Table (31): Analysis of *gus* gene transmission to the T_1 progeny in high oleic *H. annuus* L. cv.capella.

Plant number	No. of seeds	No. of germinated seeds	*Gus* expression in all germinated plants	No. of + ve PCR plants	Transgenic T_1 plants recovered
1	3	3	+	1	1/3
2	5	4	+	4	4/4
3	7	5	+	4	4/5
4	10	6	+	2	2/6
5	9	2	-	0	0

GUS was performed using random leaf of each T_1 plant.
PCR was performed with digested DNA of all plants.

Table (32): Analysis of *gus* gene transmission to the T_1 progeny in high oleic *H. annuus* L. SWSR2 inbred line.

Plant Number	No. of seeds	No. of germinated seeds	*Gus* expression in all germinated plants	No. of + ve PCR plants	Transgenic T_1 plants recovered
1	11	9	+	3	3/9
2	5	1	-	0	0
3	9	6	+	2	2/6
4	13	10	+	5	5/10
5	7	7	-	3	3/7
6	10	7	-	0	0

GUS was performed using random leaf of each T_1 plant.
PCR was performed with digested DNA of all plants.

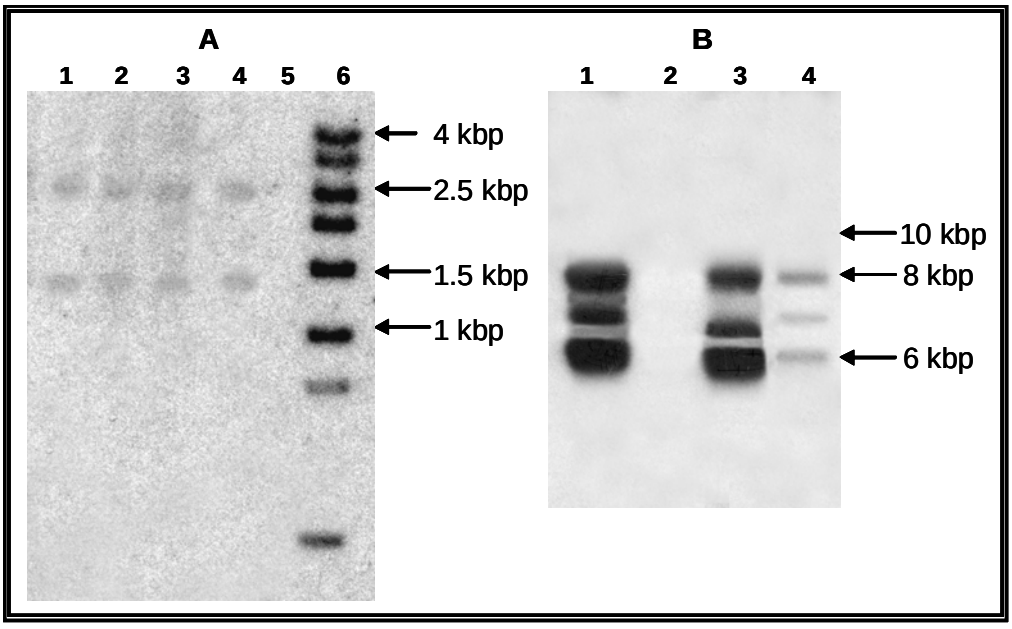

Fig. (48): Southern blot hybridization of DNA from T_1 progeny of random T_0 transgenic plants of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line transformed with *gus* gene. Genomic DNA was digested with *Hind*III and hybridised with radio labelled *gus* probe. A: cv.capella and B: SWSR2 inbred line. (A) Lane 1-4 transgenic cv.capella plants, lane 5 non-transformed cv.capella and lane 6 marker DNA. (B) Lane 1, 3, 4 transgenic SWSR2 inbred line and lane 2 non-transformed SWSR2 inbred line.

Transformation of shoot apices using *gfp* gene

The optimized transformation method for cv.capella and SWSR2 inbred line was applied in the present study to introduce another reporter gene in an effort to monitor transgene delivery to plant tissue in early developing stages and comparing the transmission of *gus* and *gfp* genes. For this aim modified *gfp* gene (*mgfp5)*, which encoding for green fluorescent protein was used in the transformation of the two high oleic *H. annuus* L. genotypes. Transformants were subjected to histological, fluorometric and molecular analysis.

❖ *Histological GFP assay*

Expression of GFP could be visually observed 20-25 days after co-cultivation or bombardment in the leaves of the regenerated shoots. GFP-expressing tissue presented bright green fluorescence, while non-expressing leaf tissue appeared dark and did not fluoresce (Fig. 49). Plantlets expressing GFP could be separated at this time and grown until maturity. The intensity of the green fluorescence detected ranged from faint to strong green. Out of 120 explants only four plants from each genotype were transgenic (Table 33).

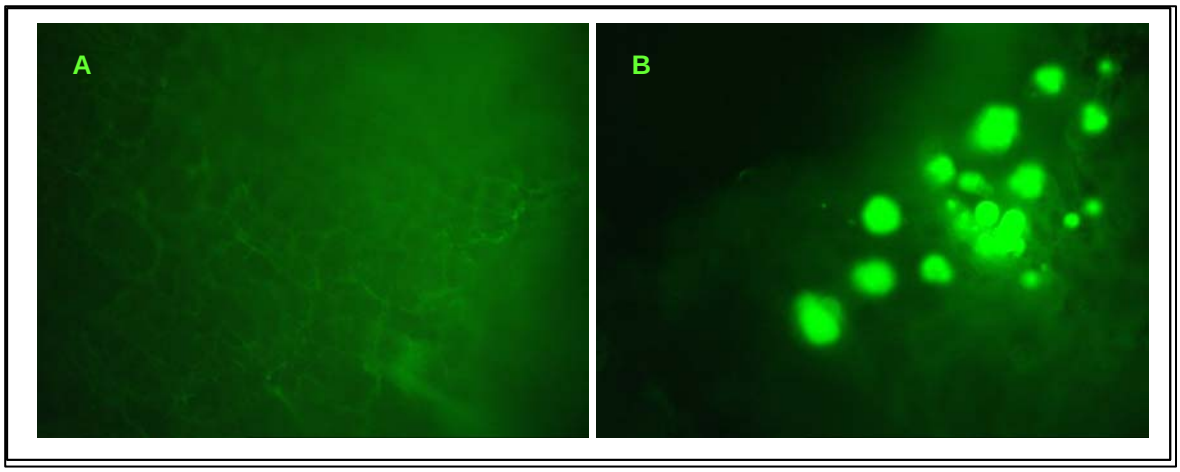

Fig. (49): Histological GFP assay of one leaf of random transgenic plant using the fluorescence microscope. (A) non-trasformed tissue and (B) positive *mgfp5*-expressing tissue.

❖ ***Fluorometric GFP assay***

Fluorometric GFP assay was performed for both the positive plants in the histological assay and non-transformed plants. The represented results in Fig. 50 confirmed the transformation in the histological positive plantlets. The intensity of the gene expression strongly varied among the transformed plants and between the two genotypes. In comparison, the maximum fluorescence value recorded in cv.capella amounted to 777.8 relative fluorescence and resulted from plant number 3, while the corresponding value in SWSR2 inbred line was 298 relative fluorescence and recorded from plant number 2.

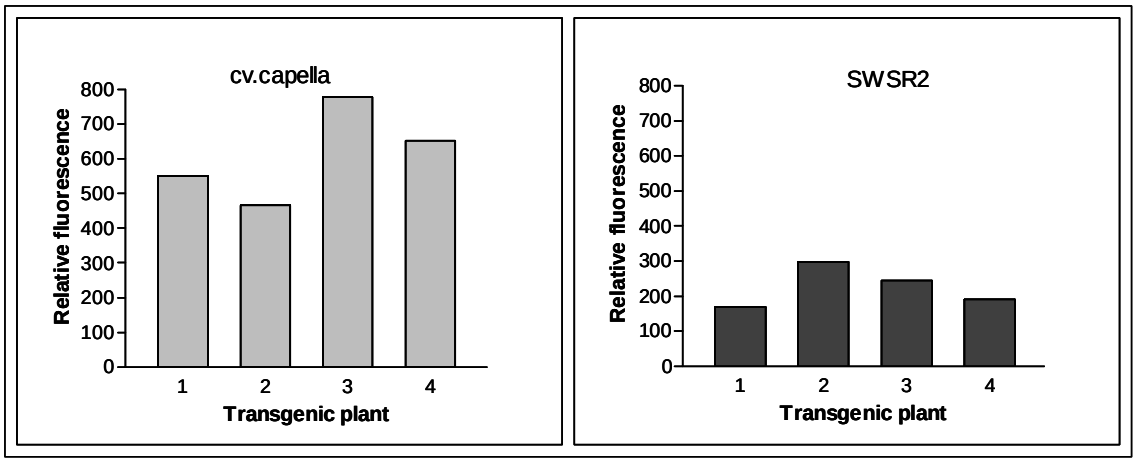

Fig. (50): Relative fluorescence of crude protein extracts from leaves of positive *gfp* transgenic plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line.

❖ ***PCR analysis of transformants***

Eventually, all the histological and fluorometric positive plantlets were also positive in PCR analysis whereas, non-expressing GFP plants showed negative results. The predicted amplified bands appeared at 800 bp using primer specific to *mgfp5* gene (Fig. 51). Transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage from the total number of co-cultivated or bombarded explants. This frequency amounted to 3.3% for both genotypes using the optimized transformation method for each (Table 33).

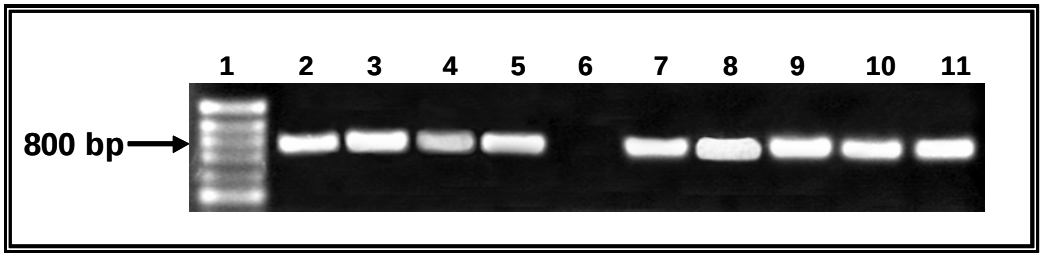

Fig. (51): PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primer to *mgfp5* gene. Lane (1) molecular marker DNA, lane (2-5) transgenic cv.capella plants, lane (6) negative control (untreated plants), lane (7) positive control (pure plasmid) and lane (8-11) transgenic SWSR2 inbred line plants.

Table (33): Summary of transformation events of split shoot apices from high oleic *H. annuus* L. genotypes cv.capella and SWSR2 using *gfp* gene.

Genotype	Total No. of used explants	No. of + ve histological assay plants	No. of tested PCR plants	No. of + ve PCR plants	Transformation frequency* (%)
cv.capella	120	4	30	4	3.3
SWSR2	120	4	30	4	3.3

(*) The percentage was calculated in relation to the total number of bombarded explants and PCR was performed with *gfp* primer 14-16 weeks after co-cultivation or bombardment. Transformation frequency was calculated on the basis of positive PCR plants.

❖ ***Southern blot analysis of transgenic plant***

To confirm that the *mgfp5* gene had stably integrated into the genome of the two tested sunflower genotypes, southern blot analysis was performed using one positive PCR plant of each genotype in addition to non-transformed sample.

Fig.52 represented the insertion events of single and two copies of *mgfp5* gene into cv.capella and SWSR2 inbred line genome, respectively (lane 4 and 3). In contrast, no band was obtained from the DNA of non-transformed sunflower (lane 1).

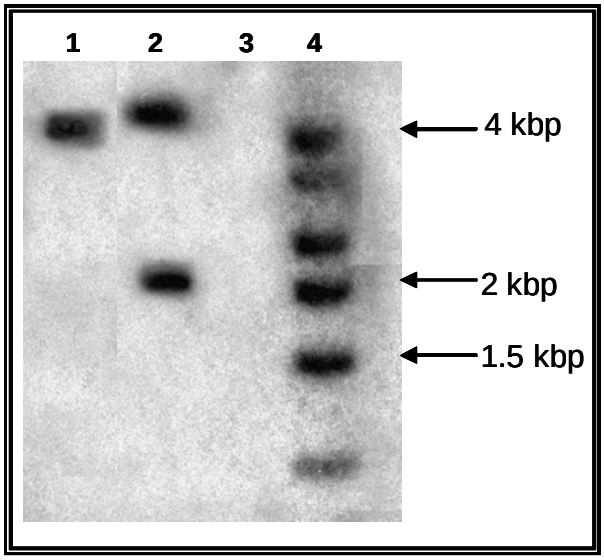

Fig. (52): Southern blot analysis of two *mgfp5* expressing T_0 plant of two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line. Genomic DNA was digested with *Eco*RI and hybridized with ^{32}P-labelled probe corresponding to *mgfp5* gene. Lane 1: transgenic cv.capella plant, lane 2: transgenic SWSR2 inbred line plant, lane 3: non-transformed sunflower and lane 4: marker DNA.

In conclusion, *gfp* gene has proved to be a suitable reporter of early transformation events. Moreover, using *gfp* gene in the transformation of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line, reduced the transformation frequencies compared to *gus* gene.

DISCUSSION

In vitro culture system

Several tissue culture variables influence regeneration events and its efficiency in sunflower shoot culture. These variables include: medium composition, sunflower genotype, types and characteristics of explant and size of the explants (Gregco *et al*., 1984; Finer, 1987, Kräuter and Friedt, 1991; Knittel *et al*., 1991; Burrus *et al*., 1991; Wingender *et al*., 1996; Bidney and Scelonge, 1997; Henn *et al*., 1998; Berrios *et al*., 1999; Müller *et al*., 2001 and Dhaka and Kothari, 2002; Yordanov *et al*., 2002).

In the present investigation two different shoot induction media (SIM1 and SIM2) were tested on different sunflower genotypes. The results confirmed that regeneration efficiency is dependent on different media components as mentioned by several reports (Jeannin *et al*., 1995, Wingender *et al*., 1996, Charrière and Hahne, 1998, Henn *et al*., 1998, Müller *et al*., 2001, Dhaka and Kothari, 2002; Yordanov *et al*., 2002). The results revealed that shoot induction was possible for all tested *H. annuus* L. genotypes on both tested media. This is due to the presence of cytokinin, which is the main organ formation stimulator, in both media (Dixon, 1987; Espinasse *et al*., 1989; Charrière and Hahne, 1998). Paterson, (1984), studied more than hundred genotypes and hybrids and demonstrated the requirement of cytokinin for shoot induction; its optimum concentration, however, varied with the genotype. Moreover, an expressive difference between media SIM1 and SIM2 was the vigor of the induced shoots; this phenomenon presumably related to the higher sucrose content and additional vitamins. It has been reported that somatic embryo formation, shoot induction or organogenesis of *H. annuus* can be easily induced, depending on the sucrose concentration of the culture medium. Additionally, the presence of vitamins in the media is known to be an important factor for differentiation or morphogenic response (Finer 1987; Jeannin *et al*. 1995; Charrière and Hahne, 1998). A negative effect of high sucrose concentration in the regeneration media can be the induction of premature *in vitro* flowering (John and Nadgauda, 1999) which is a frequent problem in sunflower regenerated plantlets (Gregco *et al*., 1984; Jeannin *et al*. 1995; Bidney and Scelone, 1997).

Analysis the response of different genotypes to different shoot induction media showed that the interaction between the genotypes and media was more pronounced in the given experiments. These results are in consistence with Gürel and Kazan, (1998) and Hewezi *et al*., (2002) who tested different genotypes, hybrids and inbred lines and got strongly different responses. Similarly, Punia and Bohorova, (1992) reported that regenerated intact plants from sunflower depend on the regeneration ability of the genotype, the nature of the explant and the hormone content of the medium.

Probably, the interaction genotype *x* media *x* tissue of the explant would be more decisive than the genetic constitution of the genotype. It means, if it is possible to combine the right media with a totipotent tissue under ideal growth conditions, the genotype will play a minor role in shoot regeneration. Several reports support this hypothesis, when they report an efficient regeneration system using a defined medium and a specific pluripotent tissue for the recalcitrant sunflower genotypes (Gregco *et al*., 1984; Finer, 1987; Burrus *et al*., 1991; Knittel *et al*., 1991; Krauter and Friedt, 1991; Krasnyanski and Menczel, 1993; Malone-Schoneberg *et al*., 1994; Wingender *et al*., 1996; Bidney and Scelonge, 1997; Charrière and Hahne, 1998; Henn *et al*., 1998; Berrios *et al*., 1999; Müller *et al*., 2001; Dhaka and Kothari, 2002; Yordanov *et al*., 2002).

The inbred line, SWSR2, on SIM2 was higher in shoot induction frequency than the hybrids because of the genetic variability and the importance of additive genetic control for organogenesis ability in some inbred lines and their F1 hybrids in sunflower. The same results were obtained by Berrios *et al*., (1999) and the same explanation was previously reported by Sarrafi *et al*., (1996a,b) and Deglene *et al*., (1997). Conversely, the regenerated shoots from cv.capella showed a superior vigor in relation to the shoots of the inbred line. This result demonstrates the heterosis effect which resulted from hybridization of un-like germplasms and appeared in F1 plants. The same heterosis effect was observed in *Pisum sativum* L. by Gregor Mendel and in sunflower by Gürel and Kasan, (1998).

Indeed genetic background of sunflower lines has been shown to greatly influence plant regeneration (Espinasse *et al*., 1989; Knittel *et al*., 1991; Nestares *et al*., 1998).

In this study, culturing of intact shoot apices induced only one shoot, while longitudinally splitting of shoot apices favoured multiple shoot induction on the explants. The multiple shoot induction after cutting the main shoots, demonstrates that the primary shoots inhibit the growth of axillary shoots. This phenomenon generally known as apical dominance (Hewezi *et al*., 2003) also proves that types and characteristics of explant play also a key role in the establishment of any regenerable tissue cultures. The obtained results agreed with Knittel *et al*., (1994) and Changhe *et al*., (2002), but are in contrast to the previous work of Gürel and Kazan, (1998) who reported that splitting the shoot apices produced fewer shoots, most likely due to the damage caused to the meristematic region by cutting with a scalpel, thus reducing the number of new shoots developing from the wounded moistens.

In addition, using split shoot apices of 4-5 mm length in the established regeneration protocol stimulate the rate of survival. Dixon, (1987) reported that size of the explants determines the rate of survival; larger shoot tip explants invariably survive better than meristems in culture.

Hyperhydratation is a very common problem in sunflower regeneration experiments. Our results showed a low incidence of hyperhydrated explants or regenerated shoots. This might be a good indication that interaction between media, growth conditions and the used tissue is suitable for efficient shoot regeneration without hyperhydratation of the explants and the induced shoots. Gibberellins can be used for the control of hyperhydratation (Fiore *et al*., 1997; Dhaka and Kothari, 2002), but generally it is very complex and involves different factors like tissue propensity and media constitution.

Presence of 0.58 μM GA3 with the elongation medium had a slight effect on shoot elongation response, whereas its high concentration promotes floral bud and callus formation in addition to rooting difficulty. These results in agreement with Paterson, (1984) who reported, with adventitious shoots regenerated from half shoot tip cultures, that flower buds would develop on shoots as soon as 3 weeks following culture and in contrast with Malone-Schoneberg *et al*., (1994), Fiore *et al*., (1997) and Dhaka and Kothari, (2002) who used other genotypes.

In vitro culture, rooting of *H. annuus* L. genotypes is a frequent problem. It is negatively affected by the early flowering response. Shoots that have flowered *in-*

vitro, are very difficult to establish in soil (Lupi *et al*., 1987; Khalid *et al*., 1992b; Biasini *et al*., 1993; Alibert *et al*., 1994).

In our protocol rooting difficulties have been overcome by directly transferring of regenerated shoots to root induction media, and then elongate the plantlets.

In attention, the root induction frequency of the hybrid was higher than in the inbred line on the two tested root induction media. The same observation was detected by Fiore *et al*., (1997) using the same RIM1. This confirms that tissue culture response in sunflower is highly genotype dependent and there is an interaction between genotypes and media (Gürel and Kazan, 1998; Hewezi *et al*., 2002).

Elongation of plantlets was performed in parallel with root system development using two shoot elongation and root development media. Viable regenerants were developed on hormone free medium (SER2) as previously reported in *Leymus chinensis* by Liu *et al*., (2004). Despite of the efficiency on shoot elongation in the SER1 medium in the presence of GA_3, we observed a higher frequency of premature *in vitro* flowering. However, from several reports we know GA_3 promotes shoot elongation and the further development of shoot buds (Power, 1987; Witrzens *et al*., 1988; Malone-schoneberg *et al*., 1994). Gibberellins applied *in-vivo* promote flowering of some species and inhibit in others. In contrary, *in vitro* gibberellins generally inhibit floral and initiate vegetative bud, but at high concentrations of GA3 in the media, our plantlets elongated, showed small leaves and the shoots became thin and slender and developed the problem of premature flowering. A similar behavior was reported by Dhaka and Kothari, (2002) for regenerated *Helianthus* plantlets. Recently, it has been demonstrated that gibberellins control meristem identity in the *Arapidopsis* and *Sinapsis* flower mutants, with flower bud induction by the activation of MADS-box genes (Li *et al*., 2002).

The regenerated plants appeared morphologically similar to mother plants. This proves that plants developed using shoot tip culture often retain the genetic composition of the mother plant (Dixon, 1987).

Phenotypically some plants were weak, sensitive, branched with flowers, and the main axis was frequently shorter than the side shoots as even reported by Knittel *et al*., (1991) and Dhaka and Kothari, (2002). This is a typical phenotype of regenerated plants related to the premature flowering *in vitro* as well as *ex vitro*.

Recently, sunflower shoot apices have been reported to bear a high potential for direct shoot regeneration (Gürel and Kazan, 1998; Dhaka and Kothari, 2002; Hewezi *et al*., 2003) and it is the only explant that has been successfully used to produce transgenic offspring (Bidney *et al*., 1992; Knittel *et al*., 1994; Grayburn and Vick, 1995; Burrus *et al*., 1996b; Weber *et al*., 2003).

In conclusion, our protocol involved direct shoot induction on the explants, thus the genetical and chromosomal variation could be avoided by the use of the meristem adjacent tissue of young shoot apices as explants for shoot induction without intervening callus phase, which is the source of somaclonal variation (Larking and Scowcroftt, 1981). These variations are undesirable when improvement is targeted using transformation technology. Furthermore, since there is no intervening callusing during the shoot differentiation process, there are chances for getting developing shoots with high genetic stability.

The number of cells or sites with regeneration potential available for the transformation per explant, in the present system, is far more than provided by the traditional leaf-disc method or cotyledonary explants.

In addition, the established regeneration protocol was repeated several times without any modification in the regeneration ability of the two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line. It is more efficient than those so far reported for the recalcitrant sunflower genotypes. Therefore, it can be set up for genetic transformation of the two high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line due to its rapidness and efficiency, since the whole regeneration process can be completed within a period of 4-6 months.

Transformation of shoot apices using *gus* reporter gene.

1- *Agrobacterium* elimination

- ***Effect of cefotaxime and carbenicillin on plant regeneration***

For effective *Agrobacterium*-mediated transformation, the antibiotic regime should control bacterial overgrowth without inhibiting the regeneration of the plant cell. For this purpose different concentrations of cefotaxime and carbenicillin were

evaluated. Our results demonstrate the diverse sensitivity of *H. annuus* L. meristematic tissue to two commonly used antibiotics for *Agrobacterium* elimination.

With regard to cefotaxime, the results showed the stimulation effect of addition of 250 mg/l cefotaxime to the shoot induction medium on shoot induction frequency as well as enhancing the characteristic of the regenerated shoots in both genotypes. Conversely, increasing the concentration to 1000 mg/l dramatically inhibited the shoot induction frequency and efficiency. These results confirmed Borrelli *et al*., (1992) results since they found that cefotaxime strongly promoted plant regeneration in two durum wheat cultivars and suggested that cefotaxime can be utilized for improving non-responding genotypes in cereal tissue culture studies. There are two possible explanations for the activity of cefotaxime in the culture, the first one is that the active molecule mimics a plant growth regulator, as suggested and discussed for hexaploid wheat and barley (Mathias and Mukasa, 1987), while the inactive form may even give toxic results. The second possible explanation that cefotaxime is converted by the cell metabolism to unknown compound with growth regulator activity Borrelli *et al*., (1992). The same positive effect of cefotaxime was observed on the frequencies of callus, root and somatic embryo formation in several *Dianthus* cultivars (Nakano and Mii, 1993).

In contrast to cefotaxime, addition of carbenicillin to the shoot induction medium had a negative effect on the shoot induction frequency and efficiency. This effect increased directly with increasing the carbenicillin concentration. Moreover, starting from 50 mg/l carbenicillin the necrosis appeared on the regenerated shoots after two weeks of induction. One of the possible reasons for carbenicillin results is that the breackdown products of antibiotics may act as growth regulators, thus modifying defined tissue culture conditions (Holford and Newbury, 1992; Lin *et al*., 1995). Another possibility is that antibiotics such as carbenicillin cause DNA hypermethylation, and therefore affect gene expression and plant development (Schmitt *et al*., 1997). Similarly, the regeneration of shoots from tobacco leaf explants was decreased as the addition of carbenicillin increased from 250 to 2000 µg/ml in the presence of 0.5 µg/ml BAP in the medium (Lin *et al*., 1995). Mihaljević *et al*., (2001) also reported that carbenicillin at 500 mg/l reduced proliferation of *P. omorika* embryogenic tissue by 50% as compared to the control and the most sensitive targets

for antibiotics are meristematic and embryogenic cells. This proves the hypothesis that some antibiotics have a detrimental effect on plant tissue cultures (Pollock *et al*., 1983; Holford and Newbury, 1992 and Lin *et al*., 1995).

In contrast, addition of carbenicillin up to 500 mg/l to *Dianthus* cultures did not appear to be toxic, but it efficiently induced somatic embryos ((Nakano and Mii, 1993).

Furthermore, Mihaljević *et al*., (2001) has compared between the effect of carbenicillin and cefotaxime at 500 mg/l on the embryonic tissue growth and revealed that cefotaxime was less toxic to the embryonic tissue growth and more suitable for *Agrobacterium* elimination.

These variant responses among the plant species and between the genotypes into the same specie could be resulted from the interaction between the genotype and the antibiotic or the mechanism of action of these antibiotics which is intimately connected to the physiological reactions of the cultured tissues (Escandón and Hahne, 1991)

Therefore, 250 mg/l cefotaxime were added to *H. annuus* L. cultures to enhance the regeneration frequency and efficiency and in the same time suppress the bacterial growth.

2- Sensitivity to selective antibiotics

❖ ***Effect of kanamycin and hygromycin on plant regeneration***

Transformation protocols require the use of a selectable marker for the identification of the rare transformants. Our marker genes are for antibiotic (kanamycin and hygromycin) resistance. It was thought that the selective agents itself might influence on the shoot induction frequency. Thus, experiments were carried out in which split shoot apices were cultured on SIM2 containing different kanamycin or hygromycin concentrations. Generally, the regeneration frequency of both genotypes was decreased as the concentration of kanamycin or hygromycin increased. The shoot induction was in pattern of initial shoots. Interestingly, at all the antibiotics concentrations, the regenerated shoots were weak, had white leaves and died after a short time. These results were also observed in both transformed and non-transformed shoots in our preliminarily transformation experiments. Moreover, starting from 2.5

mg/l hygromycin, the necrosis appeared on the regenerated shoots after 7-10 days of induction. Additionally, the high concentrations of kanamycin and hygromycin (200 and 20-40 mg/l, respectively) totally prevented shoot induction. This indicates that both antibiotics have detrimental effect on the shoot induction. There are many reports refer to the interactions between selective agent and subsequent regeneration ability (Schöpke *et al*., 1996).

With regard to hygromycin, the resulted inhibitory effect of hygromycin on shoot induction is consistent with Mihaljević *et al*., (2001) who revealed that hygromycin appeared to be highly toxic even at low concentration (2 mg/l) to *Picea omorika* embryo cultures, since it completely inhibited the induction of secondary embryogenesis. Moreover, Schrott, (1995), reported that tissues of many plant species showed higher sensitive to hygromycin than to kanamycin. In contrast, Hartman, (1991) showed that hygromycin is also a good antibiotic to use a selectable marker compared to kanamycin. This contrast may be resulted from the interactions genotype/selectable marker which should to be considered in transformation experiments Escandón and Hahne, (1991).

The detrimental effect of kanamycin was in the agreement with Escandón and Hahne, (1991), Laparra *et al*., (1995) and Müller *et al*., (2001), who used different concentrations of kanamycin for the selection of transgenic *H. annuus* L. and reported that kanamycin was found not to be a suitable marker for sunflower transformation.

In addition, the inhibition effect of kanamycin on the organogenesis was also reported in other plants such as *Malus x domestica* Borkh, *Prunus domestica* L., *Fragaria vesca* and *F.v. semperflorens* and *Pinus taeda* L. by De Bondet *et al*., (1996), Mante *et al*., (1991), Alsheikh *et al*., (2002) and Gould *et al*., (2002).

In observation, at all the kanamycin concentrations, the regenerated shoots were weak, had white leaves and died after a short time. The same observation was detected in *H. annuus* L. by Pugliiesi *et al*., (1993), *Malus* cultivars and rootstocks by Yepes and Aldwinckle, (1994b) and plum plants by Padilla *et al*., (2003). The later authors reported that once the explants exposed to kanamycin, the initiated shoots began a slow bleaching process which progressed over a several subcultures. This phenomenon was also observed in both transformed and non-transformed shoots in our preliminarily transformation experiments. One possible explanation for this

phenomenon is that kanamycin exerts its effect on mitochondria and chloroplast by impairing protein synthesis, resulting in chlorosis (Weide *et al*., 1989; Yepes and Aldwinckle, 1994b; Laine *et al*., 2000). The other explanation could be due to mutation occurrence in the explants resulted in the absence of chlorophyll in the regenerated shoots and consequently, the shoots died after a short time. The same explanation was previously suggested by Bardini *et al*., (2003) since documented that the use of kanamycin as a selective agent caused extensive methylation changes in the genome with both hyper- and hypomethylation events seen. Moreover, this effect was dosage dependent; the higher the dose, the greater the effect. At the same time, sequence mutation was detected.

Tamura *et al*., (2003) noticed escaped regenerated shoots, resulted from direct regeneration, on the selective medium which containing kanamycin up to 200 mg/l.

Furthermore, attention is increasingly directed to introduction of multiple agronomically useful genes into plant lines, without having to pyramid selectable genes in the process (Dale and Ow, 1991; Christou *et al*., 1992; Yoder and Goldsbrough, 1994; Von Bodman *et al*., 1995; Müller *et al*., 2001; Permingeat *et al*., 2003)

Considering all the previously results, we avoided to use any selective antibiotic in our transformation experiments.

3- Effect of methanol on GUS activity

Higher plants have various levels of putative endogenous GUS activities. In 1990, Hu and co-workers examined 52 plants species for intrinsic GUS-like activities and reported that such endogenous activity was detected in most of tested plants. Although, sunflower was not tested by Hu and his group, but the obtained results in this study agreed with Hu since endogenous GUS like activity was detected in the tested plants of high oleic *H. annuus* L. genotypes, namely, cv.capella and SWSR2 inbred line. The presence of the endogenous activity limits the use of the *gus* gene as a reporter gene in transgenic plants, as it may masks the activity of the introduced *gus* gene.

In our results, addition of 20% methanol to the GUS assay buffer drastically decreased the endogenous GUS like activity in both genotypes and in the same time

enhanced and activated the actual *gus* expression in the transgenic plants using some *Agrobacterium* strains. Consequently, this GUS assay method permits selective reading of the net GUS activity in transformed plant cells and prevent interference from the putative endogenous GUS activity. The same results were observed in rice and tobacco plants by Kosugi and co-workers (1990) and in sunflower by Machlab, (1996). Wilkinson *et al*., (1994), suggested that methanol does not specifically inhibit endogenous GUS like activity but it interfere with cell membrane permeability thus leading to this effect.

4- Transformation methods

The general requirements for a reliable and reproducible transformation system directed toward whole plant objectives are (i) a cell, tissue, or organ culture system capable of efficient and stable plant regeneration, (ii) a method of delivering plant expressible DNA into totipotent cells of the culture system; and (iii) the ability to identify and select regenerable cells that have been stably transformed with introduced DNA (Bidney and Scelonge, 1997; Hewezi *et al*., 2002).

On the basis of our efficient *in-vitro* culture system, various transformation methods [*Agrobacterium* infiltration, combined *Agrobacterium* infiltration with wounding systems (glass beads and microprojectiles), *Agrobacterium* injection and biolistic gene transfer] were compared.

A critical step in the development of any transformation system is the establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated. Therefore, the immediate priority was to optimize different parameters for each transformation method.

4.1- *Agrobacterium* infiltration method

Application of vacuum to plant organs in the presence of *Agrobacterium* removes intercellular fluids and air, which are replaced by bacteria when the vacuum is released (Hewezi *et al*., 2002).

❖ ***Effect of vacuum duration on split shoot apices transformation***

The achieved results from transforming split shoot apices using different vacuum duration revealed that application of the vacuum for 60 sec. increased the level of GUS expression, both in terms of the fluorometric and histochemical GUS assay. Conversely, increasing the duration above 60 sec. tends to cause reductions in the *gus* expression, shoot induction frequency and efficiency as well as the plant cell vitality. This reduction in the *gus* expression with increasing the vacuum duration perhaps is related to the reduction of the cell vitality. This explanation appears to lend credence to the hypothesis that each plant cell binds to a finite number of bacteria (Gurlitz *et al*., 1987). The same observations were detected in transformed wheat inflorescence tissue when exposed to vacuum infiltration more than 60 min. (Amoah *et al*., 2001).

❖ ***Optimization of parameters enhancing Agrobacterium infiltration method of H. annuus L. shoot apices***

The establishment of an efficient *Agrobacterium*–mediated transformation protocol for a recalcitrant crop such as sunflower requires the identification and optimization of parameters affecting T-DNA delivery and plant regeneration.

Agrobacterium strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer (Gelvin and Liu, 1994; Bhatnagar and Khurana, 2003). In addition, virulence of *Agrobacterium* strains varies widely among plant hosts (Bush and Pueppke, 1991; Davis *et al*., 1991) and is particularly important for the transformation of recalcitrant species.

Sensitivity of the two high oleic genotypes, cv.capella and SWSR2 inbred line, to different *Agrobacterium* strains was investigated. Our results showed that all the different bacterial strains tested produced transformation events with different efficiencies. LBA4404 and GV3101, carrying both the plasmid pBI121, were the most efficient strains with both genotypes. The Combination of LBA4404 strain with pBI121 plasmid has also been found to be effective for *Pinus taeda* (Tang, 2001) and *Morus indica* (Bhatnagar and Khurana, 2003). Based on *gus* expression, LBA4404 strain (octopine group) was superior and more infective than the other strains with cv.capella and GV3101 strain (nopaline group) with SWSR2 inbred line. Similarly,

LBA4404 strain was found to be significantly better than EHA105 in transformation of different cotton varieties (Sunikumar and Rathore, 2001). Nopaline strains were also infective for other plant species such as poplars (Han *et al.,* 2000). The other nopaline strain tested in this study, C58, gave low transformation efficiency. This strain harbored the plasmid pAM194 and the *gus* reporter gene under the control of *mas* promoter, while GV3101 strain has the pBI121 plasmid and *gus* gene under the 35S promoter. This means that the promoter is very important factor influencing the bacterial efficiency. These results were in accordance with Ismail *et al*., (2004) who used the same bacterial strains under the same promoters and reported that 35S promoter was more effective for *Larix decidua*. Comparing the percentage of the fluorometric assay after two and four weeks showed that a reduction in the GUS activity with the time can be seen. This indicates that the *gus* expression after two weeks was partially transient which was also previously observed in *H. annuus* L. by Hunold *et al*., (1995), who reported that *gus* expression declined with increasing culture time. The variability in the number of GUS positive shoots and expression levels was previously observed in *Lycopersicon esculentum* L. (Hamza and Chupeau, 1993), *Picea stichensis* (Drake *et al*., 1997) and *H. annuus* L. (Gürel and Kazan, 1999). Generally the different bacterial strains had a slight effect on the shoot vitality as well as on the regeneration properties.

The other important factor affecting the bacterial efficiency is bacterial density. The effect of different bacterial densities on the regeneration frequency, vitality and GUS activity was studied. Our results showed that there was an inversely proportion between bacterial density and regeneration frequency and efficiency as well as cell vitality. The reduction of the vitality, caused by inoculation with high concentration of bacterial OD_{600} (1.5 and 2.0) related to a hypersensitivity response of explants to the bacteria. The same effect of high bacterial concentration was also observed in safflower and sweet orange (Orlikowska *et al*., 1995), citrange (Changhe *et al*., 2002) and larch (Ismail *et al*., (2004). On other hand, the *gus* expression (fluorometric and histochemical) was increased in both genotypes as the bacterial concentration increased. These results were in agreement with Zhao *et al*., (2000) who reported that higher concentration of *Agrobacterium* or longer co-cultivation resulted in a high

percentage of *gus* expressing embryo in sorghum. Thus the OD_{600} value 1.0 was found to be a compromise between cell vitality and *gus* expression. The same concentration has been often used in transformation experiments of sunflower (Laparra *et al*., 1995; Lucas *et al*., 2000; Müller *et al*., 2001) and other plant species (Gutiérrez *et al*., 1997; Bond and Roose, 1998).

With regard to type of explant, we compared the split and intact explants of cv.capella and SWSR2 inbred line. The obtained results showed that there was an increase in the GUS activity with the use of split explants. Splitting of the explant facilitates the full exposure of the meristematic cells and the other tissues surrounding the meristematic region to the *Agrobacterium* infection. Another reason for increasing the *gus* expression is to increase the regeneration frequency and efficiency and subsequent gene transfer. In addition, longitudinal section through the apical meristem favoured multiple shoot induction. These results agreed with Knittel *et al*., (1994) and Malone-Schoneberg *et al*., (1994) who reported that the split shoot tip explants were more effective in the production of transgenic sunflower plants and this also was confirmed by Changhe *et al*., (2002). While are in contrast to the previous work of Gürel and Kazan, (1999), who reported that the type of shoot-tip explant (split or intact) may not be a very important factor while the existence of rapidly dividing cells in the meristem, which are potential targets for the *Agrobacterium*, is probably a more critical factor.

Among the different parameters affecting the transformation efficiency was the co-cultivation medium. Thus, two co-cultivation media (MS and YEB) were tested. The use of YEB as a co-cultivation medium increased the fluorometric and histochemical GUS activity in both genotypes compared to MS medium. These results could be resulted in excessive growth of the bacteria. Since YEB medium is known to be suitable medium for bacterial growth, it enhances the bacterial activity and hence, the transformation efficiency. Similar results were obtained by Ismail *et al.,* (2004). In contrast, Laparra *et al*., (1995) reported that any modification of the growth conditions of the agrobacteria prior to the co-culture, such as different growth media (LB, YEB, Liquid MS, or pre-culture in $MgSO_4$) was without effect on the transformation efficiency of sunflower. Conversely, YEB medium had a negative effect on the cell vitality and regeneration frequency and efficiency. This could be due to that YEB

medium is not recommended as a tissue culture medium for plants and consequently it reduces the vitality. Therefore, MS medium was selected as co-cultivation medium with respect to transformation efficiency, but without inhibiting the cell vitality and regeneration properties. MS medium has also been often used in the most sunflower transformation experiments (Müller *et al*., 2001).

There are some compounds playing an important role in the natural infection of plants by *Agrobacterium tumefaciens* because they activate the virulence genes of the Ti-plasmid and initiate the transfer of the T-DNA region into plant cell (Tang, 2001). Acetosyringone and coniferyl alcohol are phenolic compounds secreted by wounded plant cells and are known to be potent inducers of *Agrobacterium vir* genes (Stachel *et al*., 1985).

The addition of 200 µM acetosyringone during the co-cultivation increased the fluorometric GUS activity 2.8 fold in both genotypes, while addition of 100 µM coniferyl alcohol increased the fluorometric GUS activity 1.2 and 1.3 fold in cv.capella and SWSR2 inbred line, respectively, compared with the transformation without inducer. Addition of acetosyringone during pre-culture and co-cultivation increased the number of transformed cells in the target tissues in a number of species such as *H. annuus* L. (Müller *et al*., 2001), *Gossypium hirsutum* L. (Sunikumar and Rathore, 2001), *Triticum aestivum* L. (Weir *et al*., 2001) and *Larix decidua* (Ismail *et al.,* 2004). In contrast, Laparra *et al*., (1995) has reported that addition of acetosyringone (20 µM) was without effect on the transformation efficiency of *H. annuus* L. However, addition of the virulence inducers had little or no effect on the regeneration frequency in both genotypes. Therefore, we used 200 µM acetosyringone in our transformation protocol.

Pre-culture and co-cultivation duration are important factors affecting transformation efficiency and regeneration properties. Prior to inoculation with *Agrobacterium*, explants were pre-cultured on SIM2 medium for 0 to 3 days. Our results revealed that *gus* expression increased with length of pre-culture time. Improvement in transformation frequency upon pre-culturing of the explants has also been reported for *Helianthus annuus* L. (Molinier *et al*., 2002) and other plants such as *Brassica napus* L. (Cardoza and Stewart, 2003). Indeed, regeneration frequency and

shoot vitality improved with increase of pre-culture period. This means that explants were hypersensitive to the bacterial culture without any pre-culture. Consequently, a short pre-culture period (1-3 days) increased the regeneration frequency and shoot vitality because pre-culturing of the explants before co-cultivation supported explants to overcome the stress resulting from the co-cultivation with *Agrobacterium* and subsequently improving gene transfer. These results were in agreement with Molinier *et al*., (2002).

After pre-culture, the explants were transformed by co-cultivation for two and three days with *Agrobacterium*. We found that different co-cultivation times had a slight or no effect on the regeneration percentage and shoot vitality. While co-cultivating the explants for three days has been found to be better than two days in both genotypes, since enhanced the *gus* expression. These results support earlier reports in a number of species showing that the co-cultivation of the explants for three days yielded the highest transformation frequency, Chinese cabbage (Zhang *et al*., 2000), sunflower (Molinier *et al*., 2002) and wheat (Wu *et al*., 2003).

❖ ***Estimation of transformation frequency***

All the resulting optimal parameters were applied in this transformation experiment aiming estimation of transformation frequency of the method. Histochemical GUS assay results documented that the transformed tissues were uniformly expressed for the *gus* gene. The GUS frequency reached 4 and 3% in cv.capella and SWSR2 inbred line, respectively, in relation to the total number of co-cultivated explants. All the positive plants in the histochemical assay showed bands in PCR analysis. Transformation frequency was calculated on the basis of positive PCR plants and recorded as a percentage of the total number of co-cultivated explants. This transformation frequency was 4 and 3% of cv.capella and SWSR2 inbred line, respectively.

Agrobacterium infiltration method has been successfully used with other plants such as *Arabidopsis thaliana* (Poirier *et al*., 2000), *Petunia hybrida* (Tjokrokusumo *et al*., 2000), *Triticum aestivum* (Amoah *et al*., 2001), *Beta vulgaris* L. (Molnár *et al*., 2002), *Craterostigma plantagineum* (Toldi *et al*., 2002) and *Brassica napus* (Wang *et*

al., 2003). In contrast, vacuum infiltration did not give encouraging results in *H. annuus* L. transformation by Hewezi *et al*., (2002).

4.2- Wounding strategies and *Agrobacterium* infiltration method

Wounding mechanism by microprojectiles (Bidney *et al*., 1992; Malone-Schoneberg *et al*., 1994; Lucas *et al*., 2000; Hewezi *et al*., 2001) or glass beads (Grayburn and Vick, 1995) was used in an attempt to increase *Agrobacterium* transformation efficiency in sunflower. The same strategy was also reported for other plant species such as Soybean (Droste *et al*., 2000) and Sorghum (Zhao *et al*., 2000).

4.2.1- Using particle bombardment for wounding the tissue

- ***Assessment of different physical factors***

In this investigation wounding of split shoot apices with different particles sizes and particle acceleration pressures showed an general increase in the *gus* expression (fluorometric as well as histochemical) with increasing the helium pressure up to 1550 psi using either 0.4 or 0.7 µm particles size. On other hand, tissue culture performance was indeed influenced by varying helium pressure, since shoot induction frequency and efficiency as well as plant cell vitality were inversely related to the rate of helium pressure. Similarly, Bhatnagar *et al*., (2002) and Tadesse *et al*., (2003) have obtained the same results when transformed sorghum and indian mulberry via particle bombardment using gold and tungsten particles, respectively. Increase the helium pressure higher than 1550 psi caused a strong reduction in the *gus* expression and cell vitality. In addition, at a pressure of 1800 psi with either 0.4 or 0.7 µm particles size, more than 90% of the bombarded tissues of both genotypes became necrotic and formation of calli was observed. These results are in accordance with Tadesse *et al*., (2003) who reported the same observation from transformed sorghum tissues via particle bombardment. There are two possible explanations for the influence of tungsten particles coupled with different helium pressure on the regeneration properties. The first one is related to the penetration of these heavy metal particles into intact cells or tissues which may provoke various levels of tissue wounding that can have a various effect on subsequent plant regeneration (Tadesse *et al*., 2003). The second explanation could be related to the toxic effect of the tungsten particles on the

plant tissue since they often subjected to surface oxidation (Bhatnagar *et al*., 2002). This also explains the reduction of *gus* expression in the plants which transformed with more than 1550 psi helium pressure.

❖ ***Estimation of transformation frequency***

For estimating the transformation frequency of this method, all the resulting optimal parameters were applied on split shoot apices of cv.capella and SWSR2 inbred line. The histochemical assay results showed that *gus* expression was completely and uniformly in the transformed tissues with percentages of 2 and 3.3% for cv.capella and SWSR2 inbred line, respectively. The uniform *gus* expression was in agreement with Hewezi *et al*., (2001) and in contrast with Christou, (1990) and Bidney *et al.,* (1992) who reported that no uniformly transformed sunflower and soybean plants were found and the *gus* expression pattern was chimeric. Furthermore, fluorometric GUS activity results reflected the higher response of the inbred line than the hybrid for this method.

In comparison, wounding split shoot apices by microprojectiles prior to *Agrobacterium* infiltration slightly increased the transformation frequency of the inbred line compared to the *Agrobacterium* infiltration method without wounding. This strategy combines the advantages of the *Agrobacterium* with the ability of particle bombardment to generate wounds, thus enhancing the attachment of bacteria and subsequent gene transfer (Droste *et al*., 2000). However, this strategy was successfully used for other sunflower genotypes (Bidney *et al.,* 1992; Knittel *et al*., 1994, Malone-Schneberg *et al*., 1994; Lucas *et al*., 2000) and other plant species such as sugarbeet (Snyder *et al*., 1999) and soybean (Droste *et al*., 2000). Based on number PCR positive plants and related to the total number of co-cultivated explants, the transformation frequency was 3.3% for SWSR2 inbred line.

On contrary to SWSR2 inbred line, no enhancement was detected in the transformation frequency of cv.capella. The same results were also observed for sunflower (Gürel and Kasan, 1999; Hewezi *et al*., 2002) and sorghum (Zhao *et al*., 2000). This indicates that the transformation is highly genotype dependent (Gürel and Kasan, 1999).

4.2.2- Using glass beads for wounding the tissue

❖ ***Optimization of the wounding parameters***

Agitation of split shoots apices at different speeds and for different durations in the presence of *Agrobacterium* suspension resulted in a gradual reduction in the regeneration frequencies and efficiencies with increasing the speed and/or duration of the agitation for both genotypes. Moreover, a drastic reduction in the cell vitality was noticed when explants were submitted to beating at 2000 rpm. These results were consistent with Aliebert *et al*., (1999) who agitated the sunflower meristematic explants for different duration at 3800 and 4200 strokes/min. and obtained similar results.

The fluorometric and histochemical GUS activity of cv.capella decreased as the speed and/or duration of the agitation increased. The decline of GUS activity was probably due to the reduction of the cell vitality which affecting all the physiological process of the plant and subsequent gene expression.

In contrast, there was a direct proportion between the *gus* expression of the inbred line and duration of the agitation at 1000 rpm. The possible reason for this result is increase the chance for *Agrobacterium* access to the plant cell with increasing the duration of agitation. In the same time the inbred line was more tolerant to this process than the hybrid. On the whole, the most commonly observation was callus formation on the wounded explants which increased with increasing the speeds and/or durations of agitation. The typical pattern of observation on the wounded explants was reported by Tadesse *et al*., (2003).

❖ ***Estimation of transformation frequency***

In the present transformation experiment all the resulting optimal parameters were applied on split shoot apices of cv.capella and SWSR2 inbred line aiming estimation of the transformation frequency of the method. Our results showed that the transformation efficiencies varied between the tested genotypes reaching 0.9 and 1.7% for cv.capella and SWSR2 inbred line, respectively. This indicates that the transformation is highly genotype dependent and the hybrid is more responsive to *Agrobacterium* infection than the inbred line (Gürel and Kasan, 1999). Moreover, the

fluorometric result of cv.capella was 2.2 fold higher than the inbred line. Eventually, the *gus* expressing plants were positive for PCR analysis.

In comparison, application of glass bead wounding strategy prior to *Agrobacterium* infiltration reduced the transformation frequency of both genotypes than the transformation without wounding. These results probably are because of a high sensitivity of the meristematic cells to the wounding process, which interferes with long-term survival (Hewezi *et al*., 2002) or to the interaction of the bacteria with the plant cells which could be one of the determinant steps of infection process (Matthysse *et al*., 1981; Gurlitz *et al*., 1987; Winans, 1992; Sheng and Citovsky, 1996) The both sunflower publications which using glass bead wounding strategy (Grayburn and Vick, 1995; Alibert *et al*., 1999) have used young seedling, after removing the cotyledon, as explants to bypass the regeneration step (one of the most limiting step). In the established protocol we achieved, for the first time, transgenic regenerated plants of high oleic *H. annuus* L. genotypes using glass bead wounding strategy followed by *Agrobacterium* infiltration.

4.3- *Agrobacterium* injection

❖ ***Assessment of two injection capillary sizes***

Injection capillary size is a very important factor in injection process. Therefore, two injection capillary sizes were used for *Agrobacterium* injection into the split shoot apices of the two tested high oleic *H. annuus* L. genotypes. Using 7-9 µm injection capillary size resulted in a relatively low fluorometric value, while no fluorometric GUS activity was recorded from SWSR2 inbred line. When the wide capillaries (25-27 µm) were used, no *gus* expression was detected from both genotypes. The present results agreed with (Wheeler *et al*., 1991) who reported that Agroinjection has produced only a few transgenic plants

As far as the regeneration properties are concerned, using the wide injection capillaries caused a reduction in the regeneration frequency and efficiency as well as cell vitality. These effects could be due to damage of the cells inflicted by the penetrating glass pipette because of cellular pressure loss which often accompanied by a drastic change in the cellular ultrastructure followed by cell death. Interestingly, using the narrow capillaries had a slight effect on the regeneration efficiency and no effect on

the cell vitality since the narrow tip diameter inflicts substantially less damage than the wide capillaries and heat–induced expansion of the galinstan/ silicon oil filling allows fine control over the rate of injection. The similar explanation was reported by Wheeler *et al.,* (1991) and Prüfer, (2003) when explaining the most obvious drawback of microinjection process.

❖ ***Estimation of transformation frequency***

In this transformation experiment the injected regenerated plants were subjected to histochemical, fluorometric and molecular analysis. With regard to the histochemical GUS assay, the transformed cv.capella could not express the *gus* gene. Also the recorded fluorometric values of cv.capella were lower than the corresponding recorded values of other transformation methods, in addition to the high variation in the recorded values. Based on number of PCR positive plants and related to the total number of co-cultivated explants, the transformation frequency of cv.capella was 3.3% while, no transformation event was observed for SWSR2 inbred line.

Up to now, agroinjection system was used for transformation of a few plant species such as *Zea mais* L. (Escudero *et al*., 1996) and *Nicotiana benthamiana* (Hongmin *et al*., 2000). In this study Agroinjection was applied for the first time in sunflower transformation. Considering the overall results, Agroinjection method is not a recommended method for sunflower transformation. It is laborious, technically difficult and limited to the number of tissues that have been actually injected and have the regenerative abilities for the recovery of transgenic plant (Wheeler *et al*., 1991).

4.4- Biolistic gene transfer

❖ ***Evaluation of different physical and biological parameters***

Several factors have been described to influence the applicability and efficiency of biolistic gene transfer such as genotype (Koprek *et al.*, 1996), particle size (Bhat *et al.*, 2001), pre-culture period prior gene transfer (Rasco-Gaunt *et al.*, 1999), acceleration pressure (Koprek *et al.*, 1996; Bhatnagar *et al*., 2002 and Tadesse *et al*., 2003), the adjustable distances between rupture disc and target plate (Bhat *et al.*, 2001 and Rasco-Gaunt *et al.*, 1999) and number of bombardments (Lonsdale *et al.*, 1990).

For any plant tissue that is used for particle bombardment for the first time, optimal parameters must be established.

In the present investigation different physical and biological factors were optimized aiming establishment of efficient biolistic gene transfer for sunflower transformation. The obtained results showed that the highest *gus* expression values resulted from using 1.6 µm gold particles size in both genotypes. The same size of gold particle size was successfully employed in the transformation of sunflower cotyledons (Vischi *et al*., 1999), buffel grass calli (Bhat *et al.*, 2001) and sorghum shoot tip and immature embryo (Tadesse *et al*., 2003).

Changes in helium pressure were found to strongly affect the level of the gene expression. There was a directly proportion between the *gus* expression and the helium pressure up to 1550 psi. This could be related to high penetration rate of the gold particle. On other hand, the higher pressure (1800 psi) caused a dramatically reduction in the *gus* expression.

Eventually, the cell vitality was influenced by varying helium pressure and was inversely related to the helium pressure. These results explain that the damage of the cells, which increase with increasing the helium pressure, resulted in decrease the *gus* expression. Our results and explanation were in accordance with Gaunt *et al*., (1999), Bhat *et al.*, (2001), Bhatnagar *et al*., (2002) and Tadesse *et al*., (2003).

In general, increase of the target distance to 9 cm induced a reduction in the GUS activity with regardless to the particles size and the helium pressure used. Similarly, Tadesse *et al*., (2003) bombarded different types of sorghum explants at 6 cm target distance and reported that increasing the target distance could not be compensated by the elevation of acceleration pressure in any of the explants. Thus, a helium pressure of 1550 psi in combination with a target distance 6 cm and 1.6 µm particles size resulted in the highest *gus* expression frequency.

In an evaluation of the number of shots per explant, the results showed that bombardment the explants two times resulted in the highest levels of fluorometric and histochemical GUS activity. These results were in agreement with Pereira and Erickson, (1995), Schöpke *et al*., (1997) and Bhatnagar *et al*., (2002) who revealed that double bombardment of the same tissue increased the number of transformed explants.

Moreover, our results clearly demonstrated the importance of pre-culture phase which seems to be a general feature with the current technology Harwood *et al*., (1995), Hunold *et al*., (1995), Pereira and Erickson, (1995) and Nehlin *et al*., (2000). When explants were cultured for one day prior to bombardment, the highest *gus* expression frequency was obtained compared to 0 and 2 days of culture. The suggested explanation is that the increase of the *gus* expression after 1 day of pre-culture may be due to reduction of bombardment shock and consequently, tissue injury. However, shoot induction frequency and the cell vitality results were confirmed the suggested explanation since it increased as the pre-culture duration increased.

Therefore, a helium pressure of 1550 psi in combination with a target distance 6 cm, 1.6 µm gold particles size, bombardment the explants twice and pre-culture the explants for one day prior to bombardment were found to be compromise between cell vitality and *gus* expression frequency.

❖ ***Estimation of transformation frequency***

For estimation the transformation frequency of this method all the resulting optimal parameters were applied on split shoot apices of cv.capella and SWSR2 inbred line and the plants were subjected to histochemical, fluorometric and molecular analysis.

With regard to histochemical analysis results, two and one plant of cv.capella and SWSR2 inbred line, respectively, did not express the *gus* gene despite of their positive PCR results. A possible explanation for the absence of the gene expression is gene silencing which possibly resulted from the interaction among the multiple integrated copies of transgene (Sanford, 1990 and Kumpatla *et al*., 1997) or when additional copies of an endogenous gene are expressed ectopically involves homology dependent gene silencing (HDGS) (Reddy *et al*., 2003) or DNA methylation (Al-Kaff *et al*., 2000; Reddy *et al*., 2003). Similar results were previously observed in potato (Ottaviani *et al*., 1993), pearl millet (Lambé *et al*., 1995) and soybean (Reddy *et al*., 2003).

The fluorometric GUS activity and the transformation frequency results reflected that SWSR2 inbred line had a higher response than the cv.capella for the present transformation method, since the transformation frequency amounted to 3.1 and 4.5%

for cv.capella and SWSR2 inbred line, respectively. In accordance, stable transformation has been successfully achieved via particle bombardment for different plant species such as alfalfa (Pereira and Erickson, 1995), cassava (Zhang *et al*., 2000), Potato (Romano *et al*., 2001), barley (Manoharan and Dahleen, 2002), wheat (Chugh and Khurana, 2003), orchid (Men *et al*., 2003), soybean (Reddy *et al*., 2003), sorghum (Tadesse *et al*., 2003) and rice (Cho *et al*., 2004). Additionally, particle bombardment appears to be the best technique for gene transfer into conifers (Humara *et al*., 1999).

In comparison, biolistic gene transfer had achieved the highest transformation frequency for SWSR2 inbred line among other transformation methods. While the transformation frequency of cv.capella was lower than the corresponding frequency recorded by *Agrobacterium* infiltration method.

These results showed for the first time that biolistic can be successfully combined with direct regeneration system in producing stable transgenic high oleic *H. annuus* L. plants.

In summary, shoot meristems of high oleic genotypes, cv.capella and SWSR2 inbred line, were successfully employed in various transformation methods. In agreement, meristems have been previously used to produce genetically transformed plants in a range of plant species such as sunflower (Knittel *et al*., 1994; Schrammeijer *et al*., 1990; Malone-Schoneberg *et al*., 1994; Burrus *et al*., 1996b; Molinier *et al*., 2002), soybean (McCabe *et al*., 1988), maize (Lowe *et al*., 1995) and cotton (Keller *et al*., 1997).

All used transformation methods were successfully with both genotypes except agroinjection method with SWSR2 inbred line. The transformation efficiencies varied with the genotype and ranged from 1.7 to 4% for cv.capella and from 0.9 to 4.5% for SWSR2 inbred line. These results proved that the transformation is highly genotype dependent (Knittel *et al*., 1994; Gürel and Kasan, 1999). The superior transformation response of the cv.capella compared to SWSR2 inbred line in some transformation methods such as vacuum infiltration, glass bead wounding prior to vacuum infiltration and agroinjection indicates that transformation of the hybrid is easier than the inbred line. The homozygous state of the inbred line probaply leads to transformation

difficulty. These results were in agreement with Gürel and Kasan, (1999) who reported that the hybrid is more responsive to *Agrobacterium* infection than the inbred line.

Agrobacterium infiltration method and biolistic gene transfer had found to be the suitable transformation methods since they achieved the highest transformation frequency (4 and 4.5%) for cv.capella and SWSR2 inbred line, respectively. Up date, all published sunflower transformation protocols suffered from low overall transformation efficiencies which varied of 0.1% with public inbred line HA300B (Müller *et al*., 2001), 0.52% with R105 (Lucas *et al*., 2000) and 0.22% with RHA266 (Hewezi *et al*., 2002). The high achieved transformation frequency is due to the optimization of several important factors which enabled increase of the transformation frequency. This means that by fine-tuning of transformation conditions, even a recalcitrant crop like sunflower (*H. annuus* L.) can be transformed.

5- Stable transformation

Histochemical, fluorometric as well as molecular analysis confirmed that the T-DNA was integrated into the sunflower genome and transmitted to the next generation.

With regard to fluorometric analysis after different times, the obtained results showed the similarity of the gene expression in each case. There was a detectable variation in the *gus* expression into the same plant during a period of 12-14 weeks. Some plants showed stability in *gus* expression during all stages of plant development over a period of 12-14 weeks. The same observation was previously observed by Knapp *et al*., (2001) who reported that the *gus* expression was stable in transformed *Rhododendron* plants during all stages of plant development over 2 years. While in some others the *gus* expression decreased and then showed stability afterwords. This may indicate that the *gus* expression recorded 5 weeks after co-cultivation in these plants was transient expression. Moreover, another pattern of *gus* expression was detected in other plants since the *gus* expression gradually reduced with the time. This decline of *gus* expression was not due to the loss of the inserted transgene since PCR and southern analysis performed 14-16 weeks after co-cultivation confirmed the presence of *gus* sequence. Instability most probably results from transgene methylation. Similarly, several studies in plants (Linn *et al*., 1990; Kilby *et al*., 1992; Lambé *et al*., 1995) have correlated inactivation of expression with methylation of one or few

specific sites of the promoter region. These results were consistent with Ottaviani *et al*., (1993) who observed in transgenic potato plants a loss of *gus* expression contrasting with a stable resistance to kanamycin conferred by the *nptII* gene. In their study as in our study, expression of the two transgenes was driven by different promoters: the *gus* gene was dependent on the CaMV promoter and the *nptII* gene on the *nos* promoter. The different stabilities of the two transgenes reported by Ottaviani *et al*., (1993) may be linked to the use of different promoters differentially sensitive to methylation (Lambé *et al*., 1995). In addition, Hensgens *et al*., (1994) explained the same observations of *gus* expression instability in transgenic rice culture by the presence of different cell types in the culture (in which the *gus* gene is expressed or repressed) and changes in their relative contributions during subculturing. The fact that DNA methylation is known to be differentially modulated between different cell types in culture (Palmgren, *et al*., 1991) is in agreement with the hypothesis of Hensgens *et al*., (1994).

Southern blot analysis showed a single or two copies of the *gus* gene were inserted into cv.capella genome of selected T_0 and T_1 plants using *Agrobacterium* infiltration method. This pattern of insertion is very similar to the DNA insertion pattern documented for *Brassica napus* plants (Wang *et al*., 2003) using the same transformation method. However, using biolistic gene delivery has achieved multiple insertions (up to 4 copies) of the *gus* gene into SWSR2 inbred line. These multiple insertions were also previously reported in many plant species transformed via particle bombardment such as cultivated jute (Ghosh *et al*., 2002), orchid (Men *et al*., 2003) and soybean (Reddy *et al*., 2003)

The transformation frequency reported here, for the first time, for high oleic *H. annuus* L. genotypes, cv.capella (using *Agrobacterium* infiltration method) and SWSR2 inbred line (using biolistic gene transfer) was 4.1 and 4.8%, respectively. It is much higher than those have been reported for sunflower transformation. However, the higher transformation frequencies could be obtained routinely under a more optimal set of conditions (Zhao *et al*., 2000). In agreement, using *Agrobacterium* infiltration method with *Petunia hybrida* has also achieved high transformation efficiency (10% in T_1 plants) (Tjokrokusumo *et al*., 2000). Moreover, particle bombardment method has been successfully employed and achieved high transformation efficiencies amounted to

3.25% for *Hordeum vulgare* L. (Manoharan and Dahleen, 2002), 8.6% for *Triticum aestivum* var. CPAN1676, 7.5% for *T. aestivum* var. PBW343 and 4.9% *Triticum dicoccum* DDK1001, respectively (Chugh and Khurana, 2003) and 12% for *Dendrobium phalaenopsis* (Men *et al*., 2003).

Transformation of shoot apices using *gfp* gene

In this experiment introduction of *mgfp5* gene into cv.capella and SWSR2 inbred line facilitates the monitoring of the transgene in the plant tissue in early developing stages (20-25 days after co-cultivation or bombardment). This result agreed with several reports such as Jordan, (2000), Stewart, (2001) and Murray *et al*., (2004) who reported that *gfp* proved to be an excellent reporter of early transformation events. With the use of the fluorescence microscope, shoots expressing *mgfp5* gene were quickly, easily distinguished and four transgenic plants were detected of each genotype. The histological GFP assay was previously utilized by Elliott *et al*., (1999), Knapp *et al*., (2001), Müller *et al*., (2001) and Murray *et al*., (2004).

The positive plants in the histological assay were subjected to further fluorometric and molecular analysis. The fluorometric assay results showed that the intensity of the gene expression strongly varied between the two genotypes, since ranged from 466.95 to 777.75 µmol MU/mg protein/min. and from 169.0 to 298.4 µmol MU/mg protein/min. in cv.capella and SWSR2 inbred line, respectively. This also proves our previous results which revealed that the hybrid is more responsive to the transformation than the inbred line.

The transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage of the total number of co-cultivated or bombarded explants. This frequency amounted to 3.3% for both genotypes. These transformation frequencies were lower than those recorded by using *gus* gene. This decrease in frequency is probably due to the *mgfp5* gene. These results were in accordance with Murray *et al*., (2004) results. He noticed in transformation of barley cultivars that significantly reduced plant transformation frequencies were obtained with the *gfp* gene compared to *gus*.

Eventually, presence and integration of the transgene *mgfp5* in T_0 plants genome was confirmed by southern hybridization of one positive PCR plant of each genotype

in addition to non-transformed sample. Single and two copies of *mgfp5* gene were inserted into cv.capella and SWSR2 inbred line genome, respectively.

To our knowledge, the first user for *gfp* gene in sunflower transformation was Müller *et al*., (2001) who achieved stable transformation using non meristematic hypocotyls explants of public inbred HA300B with efficiency of 0.1%. In this study, for the first time, *mgfp5* gene was used in transformation of shoot apices of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line with high efficiency (3.3%).

SUMMARY

Sunflower (*Helianthus annuus* L.) is known as one of the most recalcitrant species for tissue culture and genetic transformation. The main objective of this research was to establish an efficient and reproducible transformation protocol for the production of transgenic high oleic *H. annuus* L. plants. The first step was to establish an efficient and reliable tissue culture protocol by screening diverse high oleic sunflower hybrids and inbred lines for *in-vitro* culture suitability and optimization of culture conditions for selected genotypes. For this purpose, different media for shoot induction, root induction, shoot elongation and root development were tested. On the basis of the developed tissue culture protocol, various transformation strategies [*Agrobacterium* infiltration, combined *Agrobacterium* infiltration with wounding systems (microprojectiles and glass beads), *Agrobacterium* injection and biolistic gene delivery] were compared by estimating the transformation frequency of each using the *gus* reporter gene. Several parameters affecting *Agrobacterium* infiltration method (different vacuum durations, *Agrobacterium tumefaciens* strains, bacterial densities, type of explants, co-cultivation media, virulence inducers, co-cultivation durations and pre-culture periods), microprojectiles wounding (different particle sizes and particle acceleration pressures), glass bead wounding (different speeds and durations of agitation), *Agrobacterium* injection (different injection capillary sizes), biolistic gene delivery (different gold particles sizes, particle acceleration pressures, distances between macrocarrier assembly and target plate, pre-culture durations of the explant and number of bombardments per explant) were optimized in order to determine the most efficient transformation system. These Parameters were evaluated on the basis of histochemical and fluorometric GUS activity coupled with regeneration frequency and efficiency as well as plant cell vitality. The optimized transformation protocol was applied to test the reproducibility of the method and the stability of the transmitted gene in the next generation. Further application of the optimized transformation protocol was carried out to introduce another reporter gene (*mgfp5*) in an effort to monitor transgene delivery to plant tissue in early developing stages and comparing the transmission of *gus* and *gfp* genes. Transgenic plants were subjected to

histochemical, fluorometric, histological and molecular analysis to confirm the expression, presence and integration of the transgenes in T_0 and T_1 plants.

The main important results of the present investigation are summarized as following:

- ***In vitro* culture system**

1. Cv.capella and SWSR2 inbred line were chosen among different tested genotypes because of the high frequency and efficiency of shoot induction.
2. Using split shoot apices with shoot induction medium 2 enhanced the shoot induction frequency and efficiency of selected genotypes in addition to multiple shoots formation.
3. Rooting of non elongated shoots of cv.capella and SWSR2 inbred line on root induction medium 2 induced abundant and vigorous roots with high rate (66-78%) and overcame the *in vitro* early flowering problem.
4. Shoot elongation and root development medium 2 has been found to be the suitable medium to elongate cv.capella and SWSR2 inbred line plantlets.
5. The survival frequency of the plantlets after transferring to soil was over 80% and many plants showed further development reaching the flowering stage and seed production.

- **Transformation of shoot apices using *gus* gene**

1. Addition of 250 mg/l cefotaxime to the shoot induction medium stimulated shoot induction and enhanced the characteristies of the regenerated shoots in both genotypes.
2. Kanamycin and hygromycin were found not to be suitable selection markers for sunflower transformation, thus antibiotics were avoided in our transformation experiments.
3. Addition of 20% methanol to the assay buffer for GUS activity measurements drastically decreased the endogenous GUS like activity and at the same time enhanced and activated the actual *gus* expression in the transgenic plants using some *Agrobacterium* strains.
4. Vacuum duration of 60 sec. for two times was applied in *Agrobacterium* infiltration methods as a compromise between the GUS activity and the cell vitality.

5. The two *A. tumefaciens* strains which successfully produced transformed tissues were LBA4404 strain with cv.capella and GV3101 strain with SWSR2 inbred line, carrying both the plasmid pBI121 with the *gus* gene under the control of CaMV35S promoter.
6. MS as a co-cultivation medium and OD_{600} value 1.0 were found to be a compromise between transformation efficiency, expressed as fluorometric and histochemical GUS activity, and the cell vitality.
7. Addition of 200 µM acetosyringone to the *Agrobacterium* suspension during its virulence induction period enhanced the transformation efficiency.
8. Pre-culturing the explants on the shoot induction medium for three days followed by co-cultivation with *Agrobacterium* for three days was found to be the best conditions in the established *Agrobacterium* infiltration protocol.
9. Application of the optimized *Agrobacterium* infiltration protocol resulted in 4 and 3% transformation frequency for cv.capella and SWSR2 inbred line, respectively.
10. Wounding the explants with 0.4 and 0.7 µm particles sizes at 1550 psi prior to *Agrobacterium* infiltration process yielded the highest *gus* expression levels for cv.capella and SWSR2, respectively.
11. The use of the optimal microprojectile wounding parameters slightly enhanced the transformation frequency of the inbred line from 3 to 3.3%. Conversely, reduction in the transformation frequency of the hybrid was detected compared to *Agrobacterium* infiltration without wounding.
12. Wounding the explants by agitation with glass beads in the presence of *Agrobacterium* suspension at 1000 rpm for 10 and 60 sec. produced the highest *gus* expression for cv.capella and SWSR2 inbred line, respectively, with reduction of the regeneration properties and cell vitality.
13. Using the optimized glass bead wounding strategy prior to *Agrobacterium* infiltration process reduced the transformation frequency of both genotypes compared to the transformation without wounding. However, this study is the first report about production of regenerated transgenic plants of high oleic *H. annuus* L. genotypes using this strategy.

14. Using 7-9 µm injection capillary sizes to inject *Agrobacterium* into split shoot apices resulted in relatively low fluorometric GUS activity while no *gus* expression was detected from using 25-27 µm injection capillary sizes.
15. *Agrobacterium* injection system was used for the first time in sunflower transformation and produced transgenic cv.capella plant. Meanwhile, no transformation event was observed of the inbred line.
16. A helium pressure of 1550 psi in combination with a target distance of 6 cm and 1.6 µm gold particle size resulted in the highest *gus* expression frequency which amounted to 33.3 and 30.8 % for cv.capella and SWSR2 inbred line, respectively.
17. Pre-culture the explants for one day and double bombardments of the same explant were found to be compromise between cell vitality and *gus* expression frequency.
18. On the basis of using optimized biolistic parameters, transformation frequency amounted to 3.1 and 4.5% for cv.capella and SWSR2 inbred line, respectively.
19. Comparison of the different transformation methods showed that *Agrobacterium* infiltration and biolistic gene delivery were the most efficient transformation methods since they achieved the highest transformation frequencies (4 and 4.5%) for cv.capella and SWSR2 inbred line, respectively.
20. Application of the optimized transformation protocols proved the reproducibility and reliability of these protocols and produced high transformation frequencies (4.1 and 4.8%).
21. There was a detectable variation in the fluorometric GUS activity into the same plant during a period of 12-14 weeks.
22. Histochemical, fluorometric as well as molecular analysis confirmed that the T-DNA was transferred, integrated into the sunflower genome and transmitted to the next generation.
23. Southern blot analysis showed that a single or two copies of the *gus* gene were inserted into cv.capella genome of selected T_0 and T_1 plants using *Agrobacterium* infiltration method. While using biolistic gene delivery has achieved multiple insertions (up to 4 copies) of the *gus* gene into SWSR2 inbred line.

- **Transformation of shoot apices using *gfp* gene**

1. Transformation of split shoot apices using the optimized transformation protocols combined with *mgfp5* gene facilitates the monitoring of the transgene in the plant tissue in early developing stages.
2. Using *mgfp5* gene resulted in a reduction of the transformation frequencies of both genotypes comparing with using *gus* gene. The recorded transformation frequency was 3.3% for both genotypes.
3. Presence and integration of the transgene *mgfp5* into T_0 plants genome were confirmed by histological, fluorometric and PCR analysis and southern hybridization. However, single and two copies of *mgfp5* gene were inserted into cv.capella and SWSR2 inbred line genome, respectively.
4. In the present investigation, for the first time, *mgfp5* gene has been used in transformation of shoot apices of high oleic *H. annuus* L. genotypes, cv.capella and SWSR2 inbred line with high efficiency (3.3%).

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