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PROPAGATION AND MAINTENANCE OF WILD SUNFLOWERS *in vitro*

*M. Sujatha and A.J. Prabakaran**

Division of Crop Improvement, Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500 030, INDIA

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SUMMARY

Tissue culture techniques were standardized for mass multiplication and maintenance of 18 *Helianthus* species using shoot apices and nodal explants from mature field grown plants. Meristematic explants were cultured on Murashige and Skoog's (MS) medium supplemented with varying concentrations of kinetin and benzyladenine (BA). Irrespective of the plant habit and ploidy, medium supplemented with 0.5 mg/l benzyladenine was found best for obtaining good axillary proliferation with less vitrification. Shoots from the multiplication medium were directly acclimatized bypassing the labour-intensive rooting stage. Plantlets thus obtained were successfully established in the field with a frequency of 66.5 to 100% and grown to maturity.

Key words: ***Helianthus*, nodal segments, shoot apices, tissue culture, wild sunflowers.**

INTRODUCTION

Wild sunflowers are valuable source of traits such as disease and insect resistance, drought tolerance, cytoplasmic male sterility and fertility restoration, oil quality, and early ripening (Thompson *et al.*, 1981). Conventionally these species are propagated by seeds which is often hampered by strong achene dormancy and poor seed germination (Chandler and Jan, 1985). Methods to overcome this problem by chemical, physical and physiological means/ manipulations met with little success (Heiser *et al.*, 1969; Chandler and Jan, 1985; Seiler, 1993). Furthermore, many of the species are selfincompatible and maintenance through sibbing is labour intensive. Tissue culture techniques offer a valuable tool for mass propagation and production of true-to-type plants (George, 1993). Protocols for *in vitro* propagation of cultivated sunflower using seedling shoot apices have existed for many years (Robinson and Everett, 1990) but are scanty for mature plant material of wild sunflow-

* Corresponding author

Table 1: Effect of cytokinins on morphogenic response in nodal explants of *Helianthus* species

Species	Kinetin						BA					
	0.5 mg/l		1.0 mg/l		2.0 mg/l		0.5 mg/l		1:0 mg/l		2.0 mg/l	
	No of shoots	Shoot length	No of shoots	Shoot length	No of shoots	Shoot length	No of shoots	Shoot length	No of shoots	Shoot length	No of shoots	Shoot length
H. praecox	4.8±1.5	3.2±0.8 CR	4.3±0.8	3.0±0.8 CRV	4.2±1.1	2.7±1.1CRV	17.8±4.7	2.7±0.8 CRV	9.3±2.1	2.0±0.3 CV	8.7±1.2	1.5±0.1 CV
ssp. *runyonii*	2.3±1.5	1.3±0.5 CR	1.4±0.5	2.8±1.6 CR	1.3±0.6	3.1±0.7 CR	12.3±2.2	3.2±1.2 C	9.3±2.4	1.9±0.3 C	7.8±1.5	2.1±1.1 Cv
ssp. *hirtus*	2.8±1.3	3.3±2.8	4.5±1.2	3.1±1.0 C	6.2±1.1	2.7±1.1 C	9.7±3.4	2.6±0.8 C	10.5±3.8	2.4±0.9 C	11.3±4.2	3.7±0.9 CV
H. grosseserratus	1.7±1.0	1.6±0.8 R	3.5±2.1	4.7±2.3 R	2.1±1.4	3.0±2.5	7.0±2.6	2.5±1.5	7.3±4.3	1.5±0.3	7.1±3.6	1.4±0.6
H. simulans	1.7±0.9	2.5±0.3 CR	2.8±0.8	2.0±1.4 CR	2.8±1.6	2.3±0.8 CR	7.1±5.5	1.4±0.4 C	5.3±3.5	1.0±0.5 C	4.3±2.1	1.2±0.3 C
H. niveus	6.0±2.0	4.1±0.3 RV	7.1±1.2	3.6±0.2 V	8.0±0.1	2.0±0.1 V	8.7±3.1	2.8±0.3	8.7±3.1	0.5±0.1	7.3±0.2	1.8±0.3
H. nuttallii	2.2±0.8	6.2±2.5 R	2.0±1.0	4.8±1.9 R	1.8±0.6	8.7±3.4 R	7.5±3.9	5.7±2.1 R	7.1±5.6	5.6±3.2 cR	6.4±3.8	5.4±3.1 R
H. debilis	1.3±0.5	4.0±0.1 CR	1.9±0.9	3.0±1.0 C	3.8±1.3	2.7±1.0 C	5.2±2.8	1.7±1.1 C	3.1±1.4	1.2±0.5 C	3.0±1.2	1.3±0.6 C
H. decapetalus	4.6±1.0	4.7±1.6 CRV	4.5±1.6	5.1±1.7 CRV	5.0±2.1	2.4+0.7 CV	10.2±2.0	2.0±0.8 C	11.4+3.8	2.6±0.7 C	15.0±6.3	1.3±0.4 CV
H. tuberosus	2.8±1.7	4.8±1.7 R	3.8±1.3	6.1±2.3 R	3.8±1.5	4.7±2.2 R	7.8±4.3	1.6±0.3 CR	7.3±1.1	1.9±0.5 CR	7.0±4.2	1.2±0.4 CR
H. resinosus	1.6±0.7	2.6±0.6 R	2.3±0.7	4.4±1.8 R	3.2±1.6	4.3±0.6	7.9±4.9	4.2±1.4 CR	7.0±1.4	2.7±0.6 C	6.8±2.5	2.6±0.4 C
H. annuus wild							10±1.8	3.9±1.1 R				
H. neglectus							21.7±8.4	3.2±0.6				
H. argophyllus							7.7±1.5	0.8±0.2 V				
H. petiolaris							6.2±0.7	1.9±0.1 v				
H. pauciflorus							8.6±2.8	1.8±1.1 C				
H. hirsutus							19.2±9.4	2.6±1.0				
H. strumosus							9.8±3.0	2.7±0.6				

No of shoots and shoot length are represented as means ±SE

Mean shoot length followed by letters indicates the morphogenic response; C=callusing; R=rooting; V=vitrification

ers. The present investigation has been undertaken with an aim of developing a rapid, reliable, reproducible and cost-effective protocol for *in vitro* propagation of *Helianthus* species for successful utilization in the breeding programmes for genetic improvement of cultivated sunflower.

MATERIALS AND METHODS

Plants of diploid (*H. grosseserratus, H. annuus* wild, *H. neglectus, H. argophyllus, H. simulans, H. niveus, H. praecox* ssp. *praecox, runyonii, hirtus, H. nuttallii, H. mollis, H. debilis, H. petiolaris*), tetraploid (*H. pauciflorus, H. hirsutus, H. decapetalus*) and hexaploid (*H. tuberosus, H. resinosus, H. strumosus*) wild sunflowers established in the species garden at the Directorate of Oilseeds Research, Hyderabad were used in this study. Stem segments (2 to 5 cm) possessing axillaries and shoot tips were collected from two- to four- month old plants. Explants were surface disinfested with 0.1 % (w/v) mercuric chloride for eight min. followed by four rinses in sterile glass distilled water. Nodal sections (0.5 cm) were dissected and cultured on Murashige and Skoog salt medium (1962) with 3% sucrose, 0.7% agar (Himedia) and 0.5 mg/l benzyladenine (BA) for ten days followed by transfer to the multiplication medium which consisted of 0.5 to 2.0 mg/l of kinetin and BA individually. Growth regulators were added to the medium prior to adjustment of pH to 5.6±0.1 autoclaving at 121°C for 20min. Cultures were incubated in a growth room at 26^h photoperiod provided by cool fluorescent lights (30 μmol m^{-2} s^{-1}). Regardless of rooting, proliferating shoot cultures from the multiplication medium were directly transferred to sterile vermiculite in 6 cm diameter pots after washing off the agar adhering to the roots/shoots. The pots were kept in a plastic tray and covered with a glass plate for one week after which they were transferred to the field.

Each treatment consisted of twelve culture vessels with four explants each and the experiments were conducted twice. Data were collected on the number of shoots per explant, shoot length, presence and absence of rooting, vitrification and base callusing after two weeks of culture.

RESULTS AND DISCUSSION

Nodal explants remained green with visible signs of swelling of axillaries within 7 to 10 days of culture. Transfer of the responding cultures to the multiplication medium resulted in proliferation of the pre-existing buds in the axils (Figure 1a) and callus development at the base of the explants. Base callus was green, hard and compact with the exception of *H. decapetalus* and *H. strumosus* where the callus formation was profuse, pale yellow coloured with soft texture and watery appearance. Regeneration was necessarily axillary and no adventitious regeneration was observed except in case of *H. decapetalus* and *H. strumosus* where vitrified leafy

shoots differentiated from the base callus. Cultures of *H. mollis* could not be established due to the long rosette stage and condensed nodes.

The multiplication response of different *Helianthus* species as influenced by the cytokinins is presented in Table 1. Both the cytokinins-promoted axillary proliferation and the maximum number of shoots for the different species varied between 5.2 and 21.7 per explant while maximum shoot length ranged from 0.8 to 8.7 cm. Shoot elongation in most cases was inverse to the number of shoots. In general, the multiplication rates were high on BA-supplemented media while the shoot lengths were maximum on kinetin supplemented media. However, on kinetin incorporated media the shoots were weak with thin long leaves, slender stems and long internodes. In addition to this, reculture of these shoots resulted in poor proliferation rates. Among the BA concentrations tried, medium with 0.5 mg/l BA was found to be the best as the shoots were deep green and healthy in appearance (Figure 1b) while with increasing concentrations of BA the shoots turned pale and hyperhydric. Hence, medium supplemented with 0.5 mg/l BA was used for all the species as the aim was to obtain maximum number of shoots with less vitrification and good axillary proliferation. However, an exception to this protocol was adopted for *H. argophyllus* where the vitrification was very high. In *H. argophyllus*, explant establishment and proliferation was initiated on medium supplemented with 0.2 mg/l kinetin followed by transfer to medium containing 0.5 mg/l BA where the shoot development was normal with silvery grey leaves.

Medium supplemented with 0.5 mg/l BA facilitated continuous proliferation and elongation of shoots as well. Shoot clusters were subcultured by transferring small shoot clumps directly and nodal segments of elongated shoots to fresh media every four weeks. No deterioration in shoot quality and multiplication rate was observed even after ten subcultures. A major obstacle in tissue culture of cultivated sunflower is excessive "precocious flowering" (Greco *et al.*, 1984; Paterson and Everett, 1985; Lupi *et al.*, 1987). However, in wild sunflowers including wild *H. annuus*, this problem was not encountered in spite of using explants from flowering shoots which needs detailed investigation.

Once the shoots attained maximum length and the shoot proliferative capacity ceased, rooting occurred directly or through base callus (Table 1). Rooting was most frequent on kinetin-supplemented media and in a few cases on media with lower concentrations of BA (0.5 mg/l) except for *H. nuttallii* and *H. tuberosus* where rooting consistently occurred on all the media. *In vitro*-formed roots were smooth, purple pigmented, without laterals with the exception of wild *H. annuus* where the roots were white, fragile, with laterals. Irrespective of rooting, the shoot clusters without separation into individual shoots were transferred directly to sterile vermiculite and acclimatized (Figure 1c). In cultivated sunflower, difficulties in rooting of multiple shoots is reported by several investigators (Peterson, 1984; Lupi *et al.*, 1987; Robinson *et al.*, 1987). However, this problem was not witnessed in tissue culture of wild sunflowers and the establishment of *in vitro* grown plants in soil was easily achieved (Figure 1d).

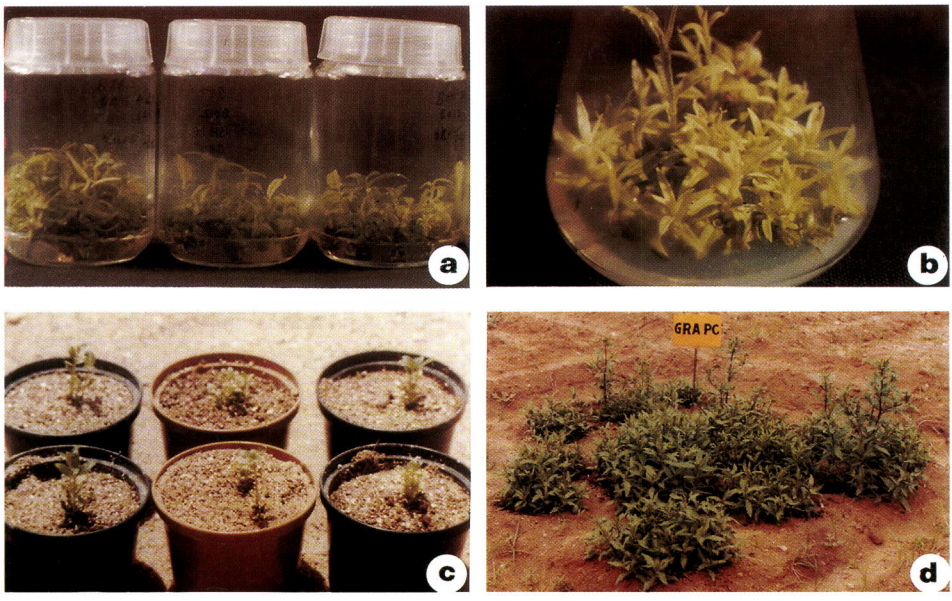

Figure 1. Propagation of wild sunflowers in vitro.
a. Proliferation of axillary buds of different wild sunflowers.
b. Healthy shoots of H. nuttallii on MS medium supplemented with 0.5 mg/l BA.
c. Acclimatization of rootless microshoots.
d. Field established plantlets of H. grosseserratus propagated through tissue culture.

After initial acclimatization, 66.7 to 100% of the plantlets developed into mature plants on transfer to the field. The survival frequencies of the perennials were comparatively higher than the annuals. Plants did not show any phenotypic abnormalities and flowered normally.

In vitro culture of plants has gained importance during recent years because, besides other applications, this technique can be used for rapid multiplication of desirable plants. For micropropagation, the explant of choice would be an apical or axillary bud to avoid any somaclonal variation (George, 1993). Tissue culture of cultivated sunflower has usually involved the use of seedling shoot apices while in the present investigation mature plant parts were utilized which enabled selection of desirable plants on the basis of phenotype. The most important aspect of this protocol is the elimination of the rooting stage and reducing the period of acclimatization, thereby significantly reducing the cost of micropropagation and also in shortening the time required to obtain a sufficient number of clonal plantlets. The protocol described is being currently used in our laboratory for maintenance of wild sunflowers and mass propagation of *Helianthus* interspecific hybrids.

REFERENCES

Chandler, J.M. and Jan, C.C., 1985. Comparison of germination techniques for wild *Helianthus* seeds. Crop Sci. 25: 356-358.

George, E.F., 1993. Plant propagation by tissue culture. Part I. The technology. Exegetics Ltd, Somerset, Great Britain.

Greco, B., Tanzarella, O.A., Carrozzo, G. and Blanco, A., 1984. Callus induction and shoot regeneration in sunflower *(Helianthus annuus* L.). Plant Sci. Left. 36: 73-77:

Heiser, C.B. Jr., Smith, D.M., Clevenger, S.B. and Martin, W.C. Jr., 1969. The North American sunflowers *(Helianthus).* Mem. Torrey Bot. Club. 22: 1-218.

Lupi, M.C., Bennici, A., Locci, F. and Gennai, D. 1987. Plantlet formation from callus and shoot-tip culture of *Helianthus annuus* (L.). Plant Cell Tiss. Organ Cult. 11: 47-55.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497.

Paterson, K.E., 1984. Shoot tip culture of *Helianthus annuus* -flowering and development of adventitious and multiple shoots. Amer. J. Bot. 71: 925-931.

Paterson, K.E. and Everett, N.P., 1985. Regeneration of *Helianthus annuus* inbred plants from callus. Plant Sci. 42: 125-132.

Robinson, K.E.P., Adams, D.O. and Lee, R.Y., 1987. Differential physiological and morphological responses of inbred lines to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid by cultured *Helianthus annuus* (sunflower) shoot tips. Plant Cell Rep. 6: 405-409.

Robinson, K.E.P. and Everett, N.P., 1990. Sunflower *(Helianthus annuus* L.*)*: Establishment of cultures, transformation, and the regeneration of plants. In: Biotechnology in Agriculture and Forestry, Vol 10 (ed: by Y.P.S. Bajaj). Springer-verlag, Berlin Heidelberg, pp. 434-452.

Seiler, G.J.,1993. Wild sunflower species germination. Helia 15: 1-8.

Thompson, T., Zimmerman, D. and Roters, C., 1981. Wild *Helianthus* as a genetic resource. Field Crops Res. 4: 333-343.

PROPAGACIÓN Y MANTENIMIENTO DE GIRASOL SILVESTRE *in vitro*

RESUMEN

Las técnicas de cultivo de tejidos fueron estandarizadas por multiplicación en masa y mantenimiento de 18 especies de *Helianthus* utilizando ápices de tallos y explantes nodales de plantas maduras en el campo. Los explantes meristimáticos fueron cultivados en un medio MS (Murashige y Skoog`s) suplementados con concentraciónes variables de kinetina y benciladenina (BA). Independientemente del hábito de crecimiento y ploidia el medio suplementado con 0.5 mg/l de BA fué encontrado el mejor para obtener una buena proliferación con menos vitrificación. Los tallos del medio de multiplicación fueron directamente aclimatizados evitando la intensiva mano de obra del estado de enraizamiento. Las plántulas obtenidas fueron establecidas con exito en el campo con una frecuencia del 66.5 a 100% y crecidas hasta madurez.

MULTIPLICATION ET MAINTIEN DES TOURNESOLS SAUVAGES *in vitro*

RÉSUMÉ

Les techniques de culture de tissus ont été standardisées pour la multiplication et le maintien à grande échelle de 18 espèces d'*Helianthus* par apex aériens ou explants nodaux, prélevés sur des plantes cultivées au champ. Des explants méristématiques sont cultivés sur un milieu de Murashigue et Skoog (MS) additionnés de diverses concentrations de kinétine et benzyladénine (BA). Indépendamment de la plante et de la ploïdie, le milieu complété par 0.5 mg/l de benzyladénine fut meilleur pour l'obtention d'une abondante prolifération axillaire et d'une vitrification réduite. Les parties aériennes issues du milieu de multiplication ont été directement acclimatées en shuntant la lourde phase d'enracinement. Les plantules ainsi obtenues sont implantées au champ avec une fréquence de réussite de 66.5 à 100% et cultivées jusqu'à maturité.