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MORFOGENETIC ABILITY OF TISSUE AND PROTOPLAST CULTURE OF WILD DIPLOID SPECIES OF SUNFLOWER (*Helianthus L.*)

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SUMMARY

An in vitro system for callus induction and plant regeneration from explants-bud, stem, leaf and cotyledon pieces as well as protoplasts isolation enzymatically from leaves and their culture in different media, is described using six wild diploid species (2n=2x=34) of sunflower viz., *Helianthus nuttallii* Tats., *H. mollis* Lam., *H. divaricatus* L., *H. debilis* Nutt., *H. maximilliani* S., and *H. praecox* E & G. All explants formed calli on a modified MS medium (MSD4). Plant regeneration was obtained in all species from calli of the above explants except cotyledons on R medium. Maximum regeneration (81.25%) was obtained from stem-induced calli of *H. nuttallii* whereas it was only 4.80% from bud-induced calli of *H. mollis*. Protoplasts from all species were examined for division, colony and subsequent callus formation. Colonies were formed in all species, however, callus formation was obtained only in *H. praecox*. Freshly isolated protoplasts of these species have shown variability for yield, size and viability.

INTRODUCTION

Sunflower being a source of seed oil and protein, is a crop of immense importance. Wild species are reservoirs of valuable genes particularly for disease, pest, drought and frost resistance, cms source, wider adaptability and profuse tillering capacity. Genetic stocks of wild species undoubtly play an important role in genetic improvement of all crop plants including sunflower.

Keeping in mind the importance of wild species, studies were undertaken to develop a system for inducing callus, its further growth and development and regeneratin of plants from the calli thus obtained from different explants, in addition to protoplasts isolation and culture. Callus regeneration of the cultivated sunflower has been reported by several authors (Sadhu, 1974; Greco *et al.*, 1984; Paterson and Everett, 1985; Lupi *et al.*, 1987; McCann *et al.*, 1988, end Espinasse *et al.*, 1989) but experiments with tissue and protolast cultures on wild species have been rather limited (Bohorova *et al.*, 1985, 1986; Punia and Bohorova, 1990). The present study will be useful for the purpose of selecting regenerative genotypes suitable for protoplast isolation, culture and fusion with cultivars of *Helianthus annuus* L.

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MATERIALS AND METHODS

Seeds of six wild diploid species (2n=2x=34) of sunflower - *Helianthus nuttallii* Tats., *H. mollis* Lam., *H. divaricatus* L., *H. debilis* Nutt., *H. maximilliani* S., and *H. praecox* E & G, were surface sterilized by stirring in 15% commercial bleach solution for 40 minutes and then rinsed 5 times in sterile distilled water. The seeds were placed aseptically on the medium BGS (Power *et al.*, 1984), for in vitro germination. Seeds were maintained at 25°C and in the light (2000 lux, day light fluorescent tubes, continous illumination). Callus induction, plant regeneration and rooting of seedlings were carried out as in the procedure described earlier by Punia and Bohorova (1990). The protoplasts from leaves of the tested species were isolated as described by Bohorova *et al.* (1986). Protoplasts were cultured in the following media viz., KP8 (Kao, 1977); MSP1, MSD4, MSZ (Power *et al.*, 1984) and B5 (Gamborg *et al.*, 1968). The size and viability of freshly isolated protoplasts were examined by light microscope.

RESULTS AND DISCUSSION

The data on callus formation (Table 1) from different explants and genotypes in MSD4 medium were quite variable. Callus formation from stem explants varied from 79.00% (*H. debilis*) to 98.00% (*H. divaricatus*) and from bud explants it ranged from 94.20% (*H. maximilliani*) to 100.00% (*H. nuttallii* and *H. debilis*). Likewise, callus formation from leaf pieces varied from 84.00% (*H. nuttallii*) to 100.00% (*H. maximilliani*), whereas, the formation was very low from cotyledon pieces of all genotypes except for *H. maximilliani* (62.00%). The results in Table 1 show that genotype, explant and medium play an important role in callus formation. The genotypes were also tested on other media but the results obtained were not encouraging. Medium MSD4 has given comparatively encouraging results not only for callus formation but also for direct regeneration from shoot tip and but; of course, it provided an ideal condition for growth and development of the regenerated plants. The callus formation in this medium requires 15 to 20 days under the cultural conditions of light and temperature described earlier.

Table 1. Mean value of callus formation (in MSD4 medium) from explants of different wild diploid *Helianthus* L. species

No	Genotype	Callus formation (%) from explant			
		Stem	Bud	Leaf	Cotyledon
1	*H. nuttallii Tats*	91.00±4.55	100.0	84.00±2.92	29.00±5.29
2	*H. mollis Lam*	82.00±7.46	98.00±2.10	93.00±2.04	47.00±6.46
3	*H. divaricatus L.*	98.00±3.30	95.50±3.40	86.00±6.76	42.00±4.28
4	*H. debilis Nutt*	79.00±9.90	100.00	92.00±4.16	27.00±4.10
5	*H. maximilliani*	84.00±6.28	94.20±3.12	100.00	62.00±3.48
6	H. praecox E & G	94.00±2.90	97.40±3.10	91.20±2.70	33.0±4.10

Well-developed calli obtained in MSD4 medium from different explants and genotypes were transferred into R medium aseptically in test tubes. The calli formed in

MSD4 medium were white to light green in colour and friable but turned dark green and non-friable in R medium. The data for regenerated calli from different explants and genotypes are given in Table 2 which reveals that callus regeneration was obtained from all genotypes and explants except cotyledons. Maximum regeneration (81.25%) was obtained from stem-induced calli of *H. nuttallii* followed by *H. divaricatus* (60.00%) and *H. praecox* (58.90%) while bud-induced calli of *H. mollis* have given the lowest regeneration (4.80%). Among leaf-induced calli, *H. praecox* has given the highest regeneration (28.60%) followed by *H. divaricatus* (18.80%).

Table 2. Mean value of regenerated calli

No	Genotype	Regenerated calli (%) inducated from explant			
		Stem	Bud	Leaf	Cotyledon
1	*H.nuttallii Tats*	81.25±6.16	11.11±1.19	16.50±1.04	0
2	*H.mollis Lam*	21.60±2.69	4.80±0.36	14.50±0.38	0
3	*H divaricatus L.*	60.00±7.16	10.51±1.01	18.80±0.92	0
4	*H debilis Nutt*	54.20±6.22	34.00±4.37	8.40±0.42	0
5	*H maximilliani*	38.00±4.27	19.40±2.11	6.80±0.47	0
6	H. praecox E & G	58.90±3.42	16.80±1.16	28.60±0.86	0

The first regeneration was obtained in 45 days, from stem-induced calli of *H. nuttallii* in R medium. The duration of regeneration varied from 45 to 80 days (from explant to regeneration) depending upon explant and genotype. So far, regeneration could not be obtained from the cotyledon-induced calli. The data for the number of embryoids per callus (Table 3) which also correspond to the number of plantlets obtained from a single callus revealed that the highest number of embryoids/callus were obtained from leaf-induced calli (9.94) of *H. preacox* and *H. maximilliani* (9.04) followed by stem-induced cali of *H. dbilis* (8.60) and *H. nuttalli* (8.80), whereas it was lowest from leaf-induced calli of *H. divcicatus* (4.12) (Figure 1).

*Table 3*Mean number of regenerated embryoids in R medium from calli of different wild diploid *Helianthus* L. species

No	Genotype	Number of embryoids*/calus from the explant			
		Stem	Bud	Leaf	Cotyledon
1	*H. nuttallii Tats*	8.50±0.90	7.24±1.42	4.78±0.34	0
2	*H. mollis Lam*	4.69±0.34	6,50±1.74	7.51±1.94	0
3	*H. divaricatus L.*	7.14±1.49	5.29±0.86	4.12±0.86	0
4	*H. debilis Nutt*	8.60±2.01	7.25±1.19	6.95±1.72	0
5	*H. maximilliani*	6.76±1.32	7.04±2.30	9.04±2.57	0
6	H. praecox E & G	7.44±0.72	5.90±1.12	9.94±1.76	0

* Als coorespond to the number of plantlets per callus

rotoplasts were readily isolated from leaves of all species investigated (Figure 2). The results in Table 4 show that the highest yield of protoplasts was from *H. mollis* (2.1 x 10^6) and *H. maximilliani* (1.9 x 10^6), with significantly lower numbers of protoplasts from the other ones. The highest mean size of protoplasts was recorded for *H. praecox* (32.4 μm). The leaf protoplasts consisted of a heterogeneous mixture of spherical cells with diameter of 7.8 to 54.6 μm. Protoplast size is an important factor from the point of plant protoplast fusion (Jones, 1988). Culture density of 2 x 10^5 protoplasts/ml was

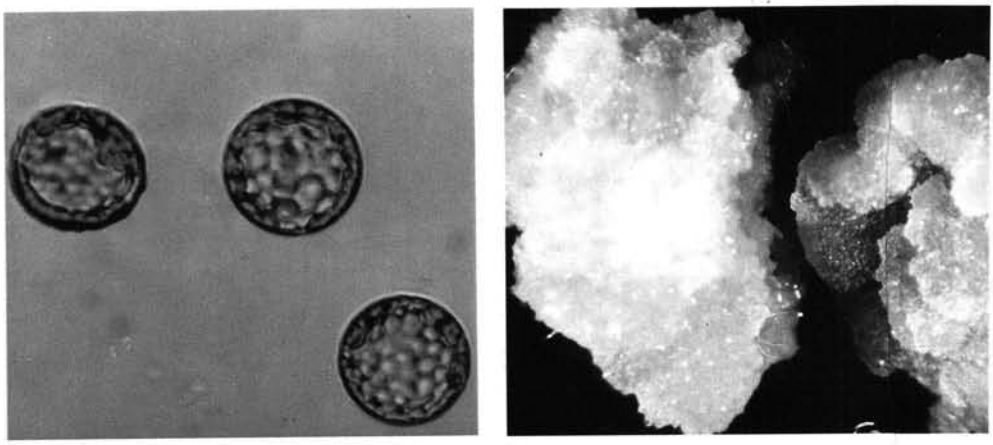

Fig. 1. Callus formation and regeneration of plantlets from stem of wild diploid Helianthus species.

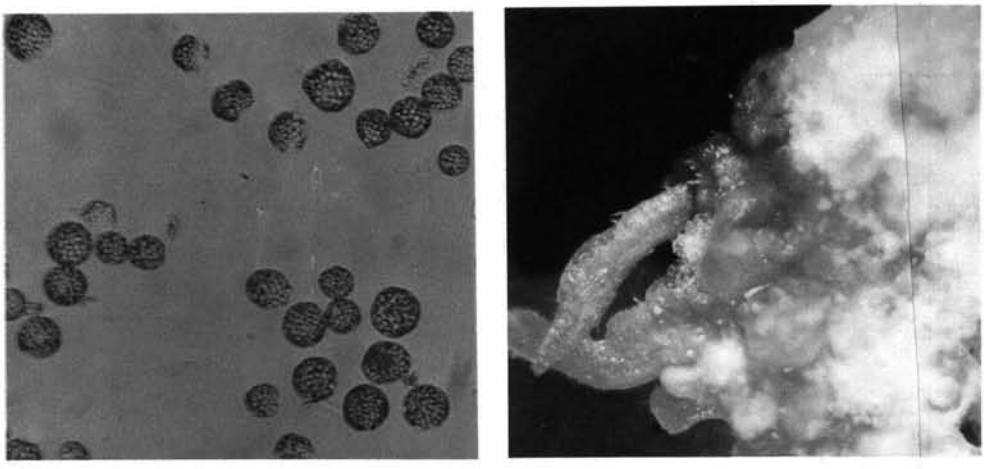

Fig. 2. Freshly isolated protoplasts from leaves of wild diploid Helianthus species.

selected as the most suitable. First division occurred within 3-5 days for all species in KPB and MSD4 liquid media, with high frequency. Protoplasts of all species have formed colonies in the above mentioned media, however, colonies from *H. praecox* grew into calli.

Our results showed that MS medium containing a cytoquinine (BAP) together with an auxin (NAA) was very effective for callus formation and its growth. These results are in accordance with the earlier finding of Bohorova *et al.*, 1986; Lupi *et al.*, 1987 and Espinasse *et al.*, 1989. This combination also helps the direct regeneration of platlets from shoot tips and buds. R medium which contains BAP, GA_3 together with camino acid has a potential for further differentiation of callus and subsequent regeneration from it. Pateson and Everett (1985) described a method of regeneration from hypocotyl-derived callus apperantely limited to a single cultivar. Bohorova *et al.* (1986) reported

Table 4. Mean yield, viability and size of leaf protoplasts from different wild diploid *Helianthus* L. species

No	Genotype	Yield of protoplasts from 1 g/FW x 10^6	Viability (%)	Size of protoplasts	
				Mean (μ)	Range (μ)
1	*H. nuttallii Tats*	1.7	98.65	26.05	7.80 –46.80
2	*H. mollis Lam*	2.1	99.25	24.88	7.80 –46.80
3	*H. divaricatus L.*	1.7	98.60	24.74	11.70–39.00
4	*H. debilis Nutt*	1.8	99.10	19.89	7.80 –39.00
5	*H. maximilliani*	1.9	98.90	29.02	11.70–46.80
6	*H. praecox E & G*	1.3	95.20	32.41	11.70–54.60

the potential for regeneration in wild *Helianthus* species. The regeneration potential in sunflower is reported to be under multigenic control with incomplete dominance (Paterson and Everett, 1985). Our results showed genotypic differences for regeneration (Table 2). The wild perennial species – *H. nuttallii* and *H. divaricatus*, had high regeneration ability. The regeneration potential of genotypes also differed with respect to explants. These differences may be due to some physiological factors. On the basis of the results obtained in protoplast and tissue culture experiments, it is obivious that all explants and genotypes differed in regeneration potential. This may be due to their individual genetic makeup which has resulted in variable regeneration ability. As protoplasts were isolated from leaves and cultured in different media, they have shown different behaviour in relation to their division, colony and subsequent callus formation which are attributable to their genetic differences. Moreover, different media have different effect on protoplast culture development which is indicative of the fact that these species differ in cultural requirements.

To the best of our knowledge, this is the first report on successful regeneration from different explan-induced calli of six wild diploid annual and perennial species of *Helianthus*, in addition to the development of colonies and calli from protoplasts of these species. Our study should give an impetus to the sunflower biotechnology, i.e., to the transfer of some valuable genes from these species to *H. annuus* cultivars through protoplast fusion as these species are likely to have high genetic potential for regeneration from protoplast cultures.

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CAPACITES MORPHOGENETIQUES DE CULTURES DE TISSUS ET DE CULTURES DE PROTOPLASTES ISSUES D'ESPECES SAUVAGES DE TOURNESOL (*Helianthus L.*)

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Un systéme in vitro visant à l'induction de callus et la régénération de plantes issus d'explants (bouton floral, tige, feuilles et morceaux de cotylédons) ainsi qu'une méthode concernant l'isolation par enzymes de protoplaste à partir de feuilles et leur culture sur différentes milieux sont présentés dans cette publication. Elle concerne six espéces sauvages diploides de tournesol: *Helianthus nuttallii Tats., H. mollis Lam., H. divaricatus L., H. debilis Nutt., H. maximilliani S.* et *H. praecox E & G.* Tous les explants ont formé des callus sur le milieu MS modifié (MSD4). La régénération des plantes a été obtenue pour toutes les especes à partir des calls issus d'explants, excepte pour les cotyledons sur milieu R. Le maximum de régénération (81,25%) a été obtenau à partir des calls induits par les tiges de *H. nuttallii*, tandis que seulement 4,80% de régénération ont été obtenues pour les calls issus de bouton floral chez *H. mollis*. Les protoplastes de toutes les espéces ont ete observés pour étudier l'apparition de division, de colonies et par la suite l'apparition de calls. Les colonies ont été formées chez toutes les especes, cependant la formation de calls n'est apparue que chez *H. praecox*. Des protoplastes fraichement isolés de ces espéces ont montré une variabilité concernant leur rendement, leur taille et leur viabilité.

APTITUD MORFOGENICA PARA CULTIVOS DE TEJIDOS Y PROTOPLASTOS DE ESPECIES SILVESTRES DIPLOIDES DE GIRASOL (*Helianthus* L.)

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Un sistema in vitro para inducción de callo y regeneración de plantas a partir de explantas yemas, tallo, hojas y trozos de cotiledones, así como el aislamiento de protoplastos enzimaticamente a partir de hojas y su cultivo en diferentes medios es descrito utilizando seis epecies diploides (2 n = 34) de girasol (*Helianthus nuttallii* tats., *H. mollis* Lam., *H. divaricatus* L., *H. debilis* Nutt., H. maximilliani S., y *H. praecox* E & G. A todos los explantes formaron callo sobre un medio MS modificado (MSD4). La regeneración de plantas se obtuvo en todas las especies a partir de callos de estas explantes excepto cotiledones en medio R. La máxima regeneración (81.25%) se obutvo a partir de callo inducido del tallo de la especie *H. muttallii* mientras que solo 4.80% a partir de callo obtenido a partir de yemas en *H. mollis*. Protoplastos de todas las especies fueron examinadas para ver división, formación de colonias y posterior formación de callo. Las colonias se formaron en todas las especies, pero la formación de callo tuvo lugar solo en *H. praecox*. Protoplastos formados de estas especies han mostrado variabilidad para rendimento, tama$_o$ y viabilidad.