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CALLUS FORMATION, EMBRYOGENESIS AND PLANTLET REGENERATION IN WILD *Helianthus* SPECIES

Gizella Németh and József Frank

Cereal Research Institute, H–6701 Szeged, P.O.Box 391, Hungary

SUMMARY

Callus cultures have been established from leaves and stem segments of perennial wild diploid species, namely *H. giganteus* L., *H. trachelifolius* Miller, *H. nuttallii* Torrey et A. Grey and *H. maximiliani* Schrad. The calli originated from leaves were weak and did not give rise to embryos. Callus growth in stem segments was intensive with $5x10^{-6}$–10^{-5}M 2,4–D and NAA. The calli were maintained in subcultures at $3x10^{-6}$M level of NAA. Regular embyoid and shoot formation was achieved with 10^{-6}M and $3x10^{-6}$M NAA in combination with BA, kinetin, zeatin and 2iP (10^{-6}–10^{-5}M). The highest number of embryoids and shoots has been obtained in all four species on solidified medium containing $3x10^{-6}$M NAA+$5x10^{-6}$M BA. In agitated liquid medium containing 10^{-6}M NAA+$5x10^{-6}$M BA abundant mass of cell aggregates was produced which in turn, plated on solidified medium with 10^{-6}M NAA+10^{-6}M BA (or kinetin or zeatin) + 40 mg/l adenine + 30 g/l sucrose, gave rise to hundreds of embryoids and shoot like structures. Rooted plants were transferred to greenhouse.

Key words: *Heliantus* species, callus formation, embryogenesys

INTRODUCTION

Wild sunflower species are potential sources of pest resistance, drouhgt tolerance, cytoplasmic male sterility and other traits that may be introduced into cultivated sunflower. For example, *H. giganteus* and *H. maximiliani* are sources of cms (Whelan, 1980, 1981) and resistance to *Plasmopara*, *Sclerotinia*, rust and *Orobanche* (Pustovoit, 1966). Also, *H. nuttallii* and *H. trachelifolius* are highly resistant to the above mentioned pests (Pustovoit, 1966).

The aspects of biotechnology in sunflower breeding have been reviewed by Friedt (1988). As for wild species, callus induction and shoot regeneration from two sterile interspecific hybrids *H. annuus* x *H. decapetalus* and *H. annuus* x *H. hirsutus* was reported by Georgieva–Todorova et al. (1980). In hard–to–cross wild sunflower species successful interspecific hybrid production was achieved by embryo culture with 53 combinations; survival of the plantlets varied between 0 and 100% (Chandler and Beard, 1983).

In 1985, sixty–five hormone combinations were tested by Bohorova et al. and successful somatic callus induction and shoot organogenesis in 3 interspecific hybrids was reported. Also, the authors obtained plantlets from anthers and immature embryos in 4 interspecific combinations. Either directly by embryogenesis or by regeneration from calli haploid, polyploid or aneuploid plants have been obtained from anthers of wild hexaplid and diploid perennial species and interspecific hybrids (Alissa et al., 1985). Successful embryo culture was reported in *H. anomalus*, *H. resinosus* and *H. anomalus* x

H. annuus (Alissa et al., 1986). In the experiments of Witrzens et al. (1988) callus cultures from tubers of *H. tuberosus* x *H. annuus* gave rise to regenerated shoots. In 46 cross combinations between cultivated and wild sunflowers 42% of excised immature embryos resulted in plantlets (Kräuter and Friedt, 1990). In two *H. petiolaris* ecotypes somatic embryo formation was induced by liquid culture from hypocotyl segments (Prado and Berville, 1990).

In six wild diploid species *H. nuttallii, H. mollis, H. divaricatus, H. debilis, H. maximiliani* and *H. praecox* successful plant regeneration was reported from bud, stem and leaf explants. In protoplast cultures of these species, division and colony formation have also been observed (Bohorova et al., 1990).

The aim of the present study was to investigate the *in vitro* morphogenetic ability of four perennial wild species involved in our breeding programme.

MATERIALS AND METHODS

Plants of perennial sunflower species *H. giganteus* L. (2n=2x=34), *H. trachelifolius* Miller (2n=2x=68), *H. nuttallii* Torrey et A. Gray (2n=2x=34) and *H. maximiliani* Schrad. (2n=2x=34) were grown in the field and upper parts (about 25 cm long) of young shoots were cut and used for experiments. Leaves and 1 cm stem segments were surface sterilized separately. After immersion in 70% ethanol for 30 second, the stem explants were surface sterilized in two steps: 5 min. with 6% Na–hypochlorite solution in semi–sterile conditions, then under laminar air flow box in sterile flasks contining 6% Na–hypochlorite (in sterile distilled water) +2 drops of Tween 80 for 10 minuter with manual shaking. Leaf explants were sterilized in the same manner but for only 5 minutes in second step. Material was thoroughly rinsed 4 times with sterilized water, then with 0,1 N HCl solution and again twice with water. In some experiments the 1cm stem segments were cut longitudinally in two halves. Leaves were cut into 1 cm wide sections.

Explants were cultured on basal nutrient medium containing macro+oligo elements of Murashige and Skoog (1962) and vitamin mixture containing (mg/l): thyamin HCL 0.25, nicotinic acid 5, pyridoxine HCL 1, Ca–DL–panthotenate 0.25, ascorbic acid 10, glycine 10, L–glutamine 50, myo–inositol 100. Depending on the experiment 20, 30 or 60 g/l sucrose was used as energy source. The pH was adjusted to 5,6 with 1 N KOH and 1 N HCl. The medium was solidified with 7 g/l Difco Bacto agar or used as liquid for suspension culture. The following plant growth regulators were used: 6–benzyladenine (BA; Sigma); kinetin (KIN; Fluka); 2–isopentenyl–adenine (2iP; Sigma); ZEATIN (Sigma); indol–3–acetic acid (IAA; Sigma); 1–naphthaleneacetic acid (NAA; Sigma); 2,4–dichlorophenoxyacetic acid (2,4–D; Sigma); indole–3–butyric acid (IBA; Sigma), adenine (Reanal). Hormones were added to medium before autoclaving at 121°C for 20 minutes. Cultures were incubated at 22°C under cool white fluorescent tubes of 40 W (2500 lux) with 12 h photoperiod.

Each combination consisted of at least 50 explants and the experiments were performed twice. Scoring was carried out in 15–day intervals with naked eye and under Wild M–8 stereomicroscope.

RESULTS

Callus induction

In each species, callus formation from leaf explants was very weak: on solidified medium containing 10^{-6}–10^{-5}M 2,4–D calli developed only at the cut surfaces and after 2 subcultures on the same hormone concentration they turned brown and died. In *H. giganteus* and *H. nuttallii*, loose, white callus layer covered about 1/4 of the original explant on medium with 0.5–1 mg/l NAA alone and supplemented with 0.5 mg/l BA or kinetin+20 g/l sucrose. In *H. trachelifolius* and *H. maximiliani* leaf cultures, white–greenish, more compact calli were formed with 0.5 mg/l NAA+0.5 mg/l BA as optimal concentration. Zeatin and 2iP (10^{-7}–$5x10^{-6}$M) had no significant effect. After four subcultures on the same medium, callus growth and proliferation stopped. No embryoid formation was observed.

Table 1. Callus formation (%) from stem explants and intensity of callus growth

Species	2,4–D, M			IAA, M			NAA, M		
	10^{-6}	$5x10^{-6}$	10^{-5}	10^{-6}	$5x10^{-6}$	10^{-5}	10^{-6}	$5x10^{-6}$	10^{-5}
H. giganteus	85.22 +	96.65 +++	84.70 ++	63.29 +	72.00 ++	90.24 ++	95.12 ++	97.89 ++++	90.19 ++++
H. maximiliani	58.09 +	90.80 +++	93.40 +++	58.53 +	76.34 +	88.46 ++	90.83 ++	93.39 ++++	94.84 +++
H. nuttallii	71.27 +	88.34 ++	93.06 +++	56.97 +	58.58 +	67.41 +	78.78 ++	89.88 +++	90.78 +++
H. trachelifolius	71.26 +	77.77 ++	79.78 +++	58.00 +	65.51 ++	73.63 ++	93.40 ++	98.97 +++	96.11 +++

Intensity of callus growth: + weak; ++ moderate; +++ intensive; ++++ very intensive

Series of auxin concentrations (0–10^{-5}M) were tested on solidified medium containing 30 g/l sucrose. There was no callus formation on hormone–free medium. Results are shown in Table 1. Callus formation took place in all the combinations after 1 week. IAA was less efficient as for the frequency and intensity of callus growth and in subcultures there was no further growth and calluses died. In all the species studied 2,4–D induced higher frequency of callus formation but until the 3rd subculture calli survived better at $5x10^{-6}$M. Very high callus formation frequency was observed with NAA where the $5x10^{-6}$M (or in later experiments $3x10^{-6}$M) concentration gave rise to white-greenish loose and/or compact callus tissues (Figure 1 a, b). There was no significant difference for frequency and callus growth intensity between whole segments and those cut into two halves. For further work calli subcultured at $3x10^{-6}$M NAA were used.

Embryogenesis and plantlet regeneration

In the experiments aimed at the induction of organogenesis, 10^{-6}M and $3x10^{-6}$M NAA were used in combination with different cytokinins such as BA, kinetin, zeatin and 2iP (10^{-6}, $6x10^{-6}$ and 10^{-5}M). On hormone–free medium, frequency of embryoid formation was low: from 0.5 to 1,6 embryoid/callus and they did not develop into plantlets. In all the combinations of NAA+cytokinin embryoid formation took place in most of the calluses (51–80% of them were embryogenic) of the four species. Their size varied between 0,5 and 2 mm and larger ones had tiny leaf primordia and/or leaves. In many cases it was difficult to count them because of the presence of more developed shoots. In such cultures callus was covered with green(ish) structures. Presumably, direct embryogenesis

a) *b)*

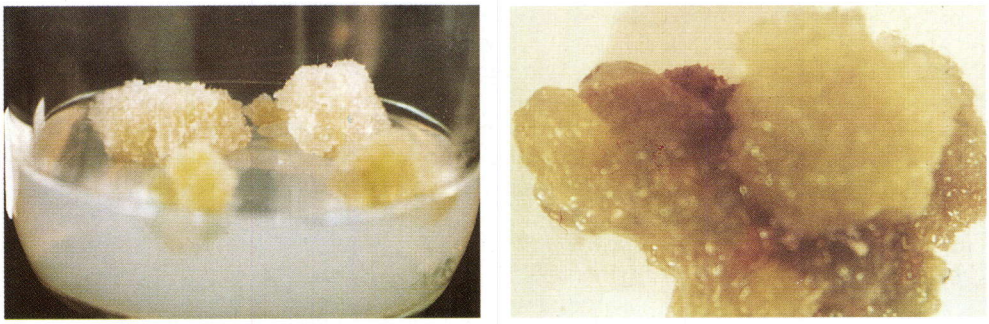

Figure 1. Callus formation from stem segments of a) H. maximiliani; b) H. giganteus at 5x10^{-6}M NAA

and shoot formation occurred simultaneously in calluses. Sometimes, callus formation was less intensive and embryos were observed on the surface of explant itself. Results are presented in Table 2 and in Figure 2. to Figure 5. The more efficient concentration of cytokinins was found to be 5x10^{-6}M and the highest number of embryoids and shoot(let)s was obtained in combination with 3x10^{-6}M NAA. The most efficient cytokinis were BA and kinetin, zeatin and 2iP were less effective. *H. giganteus* and *H. maximiliani* responded with higher number of embryos and shoots than *H. nuttallii* and *H. trachelifolius*. At 10^{-5}M cytokinin concentration the calluses became more compact and the size of embryoids was smaller; shootlets from these calluses were morphologically distorted and often showed vitrification.

Table 2. Average number of embryoids (E) and shoots (S) per callus in the presence of 3x10^{-6}M NAA + 5x10^{-6}M cytokinin

Cytokinin / Species	BA		KIN		Zeatin		2iP	
	E	S	E	S	E	S	E	S
H. giganteus	7,45	3,4	6,31	2,9	5,3	2,4	4,2	1,8
H. maximiliani	8,3	4,5	8,1	4,6	7,4	3,8	4,2	2,1
H. nuttallii	6,2	3,1	5,8	3,4	5,5	2,5	4,1	1,8
H. trachelifolius	6,9	3,8	6,3	4,0	5,4	3,1	5,0	2,7

Calli of *H. giganteus, H. maximiliani* and *H. nuttallii* were cultured also in agitated liquid medium at 10^{-6}M NAA + 5x10^{-6}M BA. After one week the cultures consisted of cell aggregates of 1–3 mm size and in almost each "crumb" a green region appeared. Subcultures were made weekly and after 1 month the supensions were plated in jam jars to solidified medium containing 30 g/l sucrose + 40 mg/l adenine + 10^{-6}M NAA + 5x10^{-6}M kinetin or BA or zeatin. Two weeks later the surface of the medium was covered with dense mass of "crumbs" white and green in colour which represented embryoids and shoot–like structures.

A partion of shootlets was separated from the callus and transferred to a rooting medium with no auxin or with 10^{-6}M IBA. Rooted plantlets were obtained and planted into greenhouse under plastic cover.

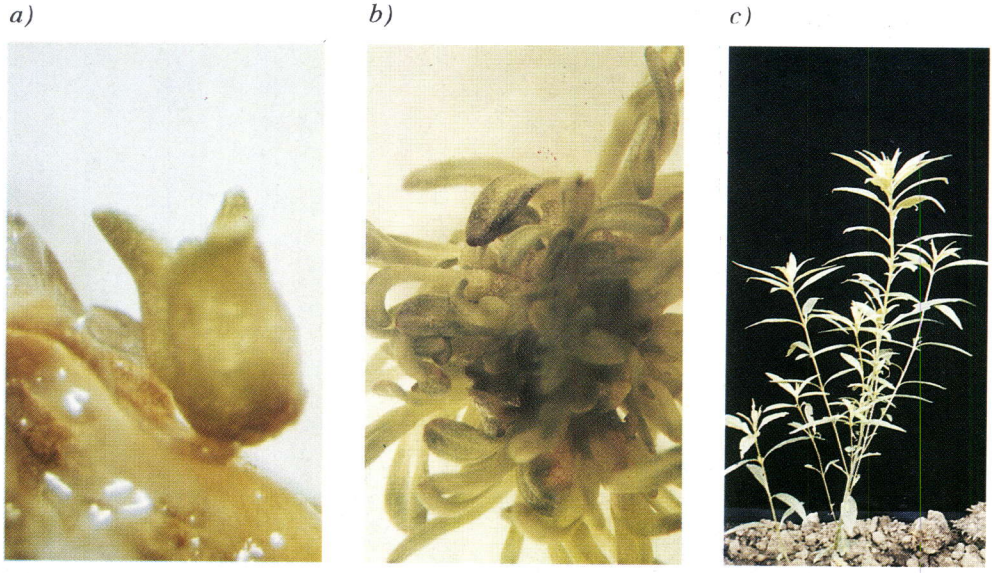

Figure 2. H. giganteus: a) embryo emerging from the surface of stem explant, b) abundant shoot formation; c) regenerated plant from callus transferred to greenhouse

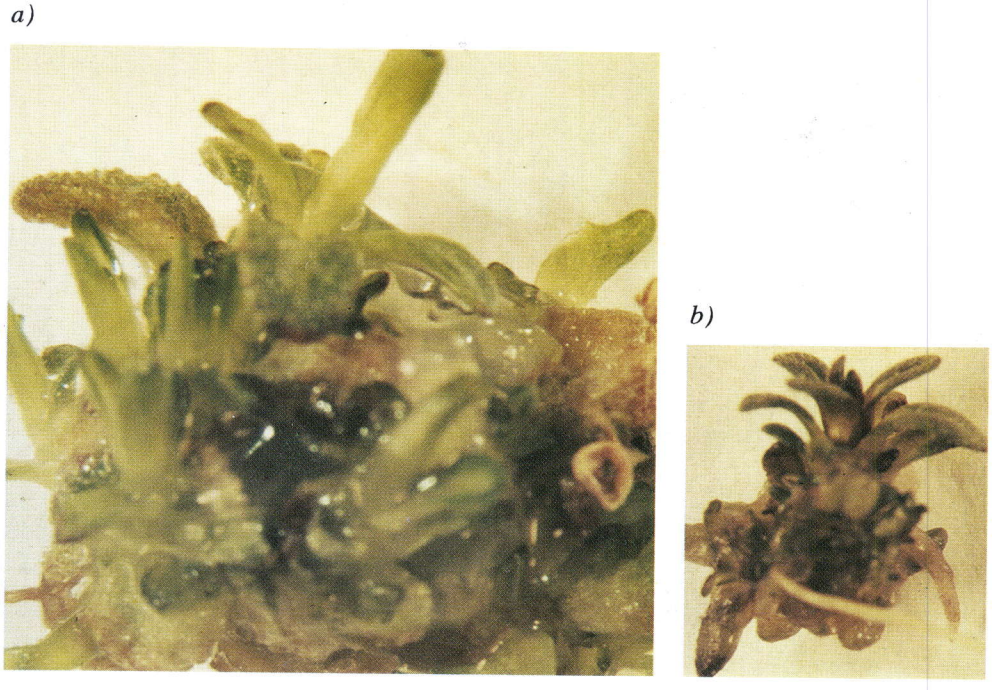

Figure 3. H. trachelifolius: a) shoot formation from embryos; b) rooted shoot

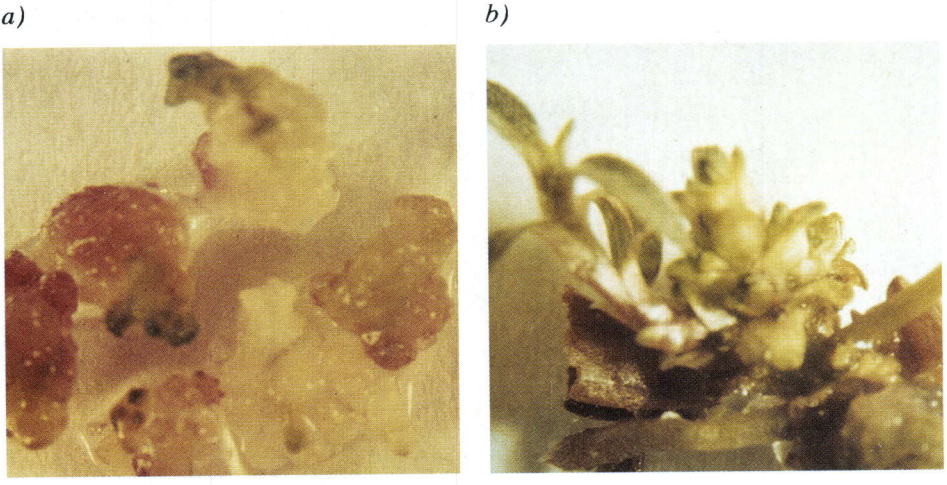

Figure 4. H. maximiliani: a) embryo(id) formation from cell aggregates; b) direct embryo and shoot development from explant

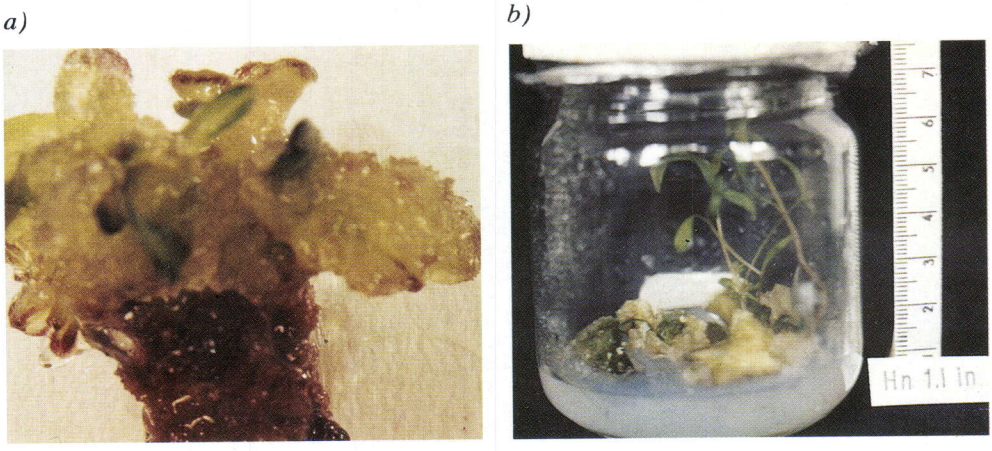

Figure 5. H. nuttallii: a) embryogenesis in callus; b) callus and regenerated plantlet

DISCUSSION

Callus forming ability of leaves was very low with 2,4–D and the calli did not survive. It reminds of the negative effect of 2,4-D on cultured anthers (Alissa et al., 1985). However, the callusing was moderate or even intensive in stem explants with *H. annuus* (unpublished) and with those of Bohorova et al. (1985). Since the calli were less vigorous and in subcultures their growth slowed down and many of them died, we did not continue further experiments in spite of embryo inducing ability of 2,4–D reported by Dupuis et al. (1988) and Prado and Berville (1990). IAA proved to be a less efficient auxin. In the majority of publications NAA was reported as a powerful auxin which in combinations with BA induced embryogenesis and shoot development from calluses (Bohorova et al., 1985, 1990); Paterson and Everett, 1985; Witrzens et al., 1988). In all 4 species studied, BA and kinetin induceda higher number of embryos than zeatin and 2iP that might be due to their higher activity. In liquid culture 40 mg/l adenine was added to the medium and in some other experiments it has been tried together with cytokinin but we did not find it essential as it was stated by Bohorova et al. (1985). Callus formation and subsequent embryoid induction was high in *H. maximiliani* and *H. nuttallii* which is in coincidence with the data of Bohorova et al. (1990), although, we had no success with leaf cultures. This failure may also be due to the age and physiological state of leaves. Genotypic differences did not seem to play a significant role in embryo formation, although in callus induction there were some differences which may also be due to vigor of the source material.

CONCLUSIONS

In *H. giganteus, H. maximiliani, H. nuttallii* and *H. trachelifolius,* intensive callus growht was achieved with subsequent embryo and shoot formation in the presence of NAA and different cytokinins. Viable rooted plantlets have been obtained. Thus, potentially, somaclonal variants may be produced *in vitro* as a source of genetic material.

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FORMACION DE CALLO, EMBRIOGONESIS Y REGENERACION DE PLANTULAS EN ESPECIES SILVESTRES DE GIRASOL

RESUMEN

Cultivos de callo han sido establecidos a partir de hojas y segmentos de tallos de las especies silvestres diploides perennes, H. *gigantens* L., H. *trachalifolius* Miller, H. *nuttallii* Torrey et A. Grey y H. *maximiliani* Schrad. Los callos originados a partir de las hojas fueron débiles y nodieron lugar a embriones. El crecimiento de callo en segmentos de tallo fue intensivo con $5x10^{-6}$–10^{-5} M2, 4–D y NAA. Los callos fueron matenidos en subcultivos al nivel de $3x10^{-6}$ M de NAA. Una formación regular de embrioides y tallo fue alcanzada con 10^{-6} y $3x10^{-5}$ M, NAA en combinación con BA, kinetina, reatina y 2iP (10^{-6}–10^{-5} M). El número mas alto de embrioides y tallos ha sido obtenido en las cuatro especies en un medio sólido conteniendo 10^{-6} M NAA +$5x10^{-6}$ M BA. En medio líquido agitado conteniendo 10^{-6} M NAA +$5x10^{-6}$ M BA. Se produjo una abundante masa de agregados de células que sucesivamente, colocada en placas con medio solidificado 10^{-6} M NAA +5 x 10^{-6} M BA (o Kinetina o zeatina) + 40 mg/l adenina + 30 g/l sucrosa, dieron lugar a cientos de embrioides y estructuras de tallos. Las plantas enraizadas fueron transferidas al invernadero.

FORMATION DE CALLUS, EMBRYOGÉNÉSE ET RÉGÉNÉRATION DE PLANTULES CHEZ LES ESPÉCES SAUVAGES DE TOURNESOL.

RÉSUMÉ:

Les cultures de callus ont été obtenues à partir de feuilles et de segments de tiges des espéces sauvages, luriannuelles diploïdes suivantes: *Helianthus giganteus* L.,*Helianthus trachefolius* Miller, *Helianthus nutallii* Torrey et A.Grey et *Helianthus maximiliani* Schrad. Les callus obtenus à partir des feuilles étaient faibles et n'ont pas donné lieu à la formation d'embryons. Les calls issus des segments de tige étaient nombreux pour des concentrations de 5 x 10^{-6} - 10^{-5} M2,4-D et NAA. Une formation régulière d'embryons et de racines a été obtenue avec 10^{-6} M et 3 x 10^{-6} M NAA en associassion avec BA, kinetin , zeatin et 2iP (10^{-6}M et 3 x 10^{-5}). le nombre le plus important d'embryons a été obtenu pour les quatre espéces sur des milieux solidifiés contenant 3 x 10^{-6} M NAA et 5 x 10^{-6} M BA. Sur des milieux agités tittrant 10^{-6} M NAA et 5 x 10^{-6} M BA une abondante masse de cellules se forme qui semées sur un milieu solide avec 10^{-6} M NAA, 5 x 10^{-6} M BA (ou kinetin ou zeatin) , 40 mg/l d'adénine et 30 g/l de saccharose ont donné lieu à la formation de centaine d'embryons et de structure de type racinaire. Les plantes racinées ont été transferrées en serre.