

## ANDROGENETIC RESPONSE OF SUNFLOWER IN DIFFERENT CULTURE ENVIRONMENTS

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### SUMMARY

Production of microspore-derived embryos from cultured anthers is now a well-established technique for the isolation of homozygous lines in sunflower. Experiment was conducted to optimize the culture composition for wild *Helianthus* and interspecific hybrids. Anthers of uninucleate microspores were cultured in basal MS media with different hormonal combinations. The induction of callus was quick and proliferation high with the presence of 2,4-D, low cytokinin and auxin contents. Excess cytokinin and auxin had no significance in callus induction. Regeneration potential increased with the increased/optimum amount of casein hydrolysate and benzyl amino purine. Kinetin had no specific influence of regeneration frequency of callus. Genotype had a significant effect on the capacity of callus induction and plantlet formation. *H.annuus* × *H.tuberosus* responded well and produced plantlets in optimum culture medium. Androgenesis of interspecific hybrid *H.annuus* × *H.tuberosus* formed plantlets that surpassed the embryoid stage.

**Key words:** *Helianthus*, sunflower anther culture, doubled haploids – culture media

### INTRODUCTION

Wild sunflowers are valuable sources of traits such as disease and insect resistance and drought tolerance (Thompson *et al.*, 1981). The introgression of alien genes from wild into the cultivated form and the development of inbred lines require a long breeding cycle. As an alternative to conventional breeding, homozygous diploid lines can be obtained *via* androgenetic haploids in a relatively short period of time. Such doubled haploids from interspecific hybrids (Chandler and Beard, 1983) could be useful sources for the screening of (recessive) genes for disease resistance from wild *Helianthus* species. Crosses between *H.annuus* (L.) and wild relatives, which are often partially or completely sterile, are used as donor plants for anther or microspore culture. The advantages would be genomic reduc-

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tion and improved fertility, useful for trait introgression into the cultivated sunflower genome (Zhong *et al.*, 1995). Haploids of sunflower have already been obtained via anther culture (Alissa *et al.*, 1985, Bohorova *et al.*, 1985). Though androgenesis is a valuable tool in speeding up the progress of crop improvement, its success lies in the ability to manipulate anthers and microspores *in vitro* in a way that a wide array of genotypes responds with high regeneration frequency (Thengane *et al.*, 1994). Low-frequency shoot generation from anther callus has been reported in some interspecific hybrids of sunflower. The rate of callusing was dependent on genotype, nutrient medium and stage of explant (Vasiljević *et al.*, 1990). The present paper describes further investigations aimed at optimizing culture conditions facilitating high frequency of androgenesis in sunflower.

## MATERIALS AND METHODS

### Plant material

A total of six genotypes were used in the experiment, the cultivated inbred "Morden", three wild forms viz., *H.occidentalis* (2×), *H.decapetalus* (4×) and *H.tuberosus* (6×), and two interspecific hybrids (Morden × *H.tuberosus*, Morden × *H.occidentalis*). The plants were grown in a greenhouse, Tamil Nadu Agricultural University, Coimbatore, during 1999-2001 without any temperature control.

### Anther stage and collection

The flower buds of 1.5 to 2.0 cm in diameter for Morden and 0.5 to 0.7 cm in diameter for *H.occidentalis*, *H.decapetalus* and *H.tuberosus* with cream-colored anthers possessing mostly uninucleate microspores were used. The flower buds were surface sterilized by immersing in 70% ethanol and 2.5% mercuric chloride for 2 minutes each. After each treatment, buds were rinsed twice in sterile distilled water. The anthers which were at the mid- to late uninucleate stage were excised under a stereo binocular microscope. All extraneous materials like papillae were removed. The white conical ovary portion at the bottom and the corolla joint at the top were cut with the help of a sharp scalpel to squeeze out the bundle of syngeneisous anthers.

### Culture initiation

The basal medium of Murashige and Skoog (1962) with organic supplements like casein hydrolysate was used in all experiments. It was supplemented with growth hormones at several concentrations (Table 1). All media contained 30 g/l of sucrose and were solidified with 8 g/l of bactoagar. The pH of the media was adjusted to 5.6 to 5.8 using 0.1 N HCl or 0.1 N NaOH. 15 ml of medium was poured in 25 × 125 mm size culture tube. The culture tubes were plugged with sterile non-absorbent cotton and autoclaved at a pressure of 20 pounds / sq inch for 15 minutes at 21°C. Five anthers were inoculated in each culture tube and maintained at

25°C with 16 hours light period of 3000 lux and eight hours dark. The friable anther calli developed in different treatments (AC<sub>1</sub> to AC<sub>7</sub>) were transferred to the same callus induction medium after 20-25 days.

Table 1: Effect of MS + additives on anther callus induction

Treatment	Basal	CH (mg l <sup>-1</sup> )	Auxin (mg l <sup>-1</sup> )		Cytokinin BAP (mg l <sup>-1</sup> )
			NAA	2,4 - D	
AC1	MS	0	0.0	0.0	0.0
AC2	MS	0	0.1	0.0	0.2
AC3	MS	0	2.0	0.0	1.0
AC4	MS	250	1.0	0.0	0.5
AC5	MS	250	1.0	2.0	0.5
AC6	MS	250	2.0	0.1	1.0
AC7	MS	500	2.0	0.0	1.0

### Regeneration

The friable embryogenic calli from the best callus from culture induction media were transferred to nine different media combinations (Table 2) with basal MS medium after 30-40 days of plating depending on proliferation. The medium was maintained in the same condition as done in culture initiation.

Table 2: Effect of MS + additives on anther callus regeneration

Treatment	Basal	CH (mg l <sup>-1</sup> )	BAP (mg l <sup>-1</sup> )	Kin (mg l <sup>-1</sup> )
AR1	MS	100	0.2	0.0
AR2	MS	100	0.5	0.0
AR3	MS	100	0.5	0.5
AR4	MS	250	0.2	0.0
AR5	MS	250	0.5	0.0
AR6	MS	250	0.5	0.5
AR7	MS	500	0.2	0.0
AR8	MS	500	0.5	0.0
AR9	MS	500	0.5	0.5

Each treatment consisted of three culture tubes with five explants each and the experiments were conducted twice. Data were collected on callus induction percentage, days to callus induction, days to maximum callus proliferation, nature of callus induced and regenerative calli from anther was measured. The observations were statistically analyzed using simple statistics.

## RESULTS

The anthers turned brown after 3-5 days from plating and callus initiations was observed on different days on the two ends of anthers. The treatment AC5 gave maximum response for callus induction for most of the genotypes and AC2 ranked second for maximum callus induction response (Table 3). Days to callus initiation

ranged between 5 and 10 days. It varied with the genotype and the media composition. For all genotypes, the treatment AC4 recorded minimum time for callus initiation and proliferation followed by AC2. Maximum time was taken by AC 7 (Table 4).

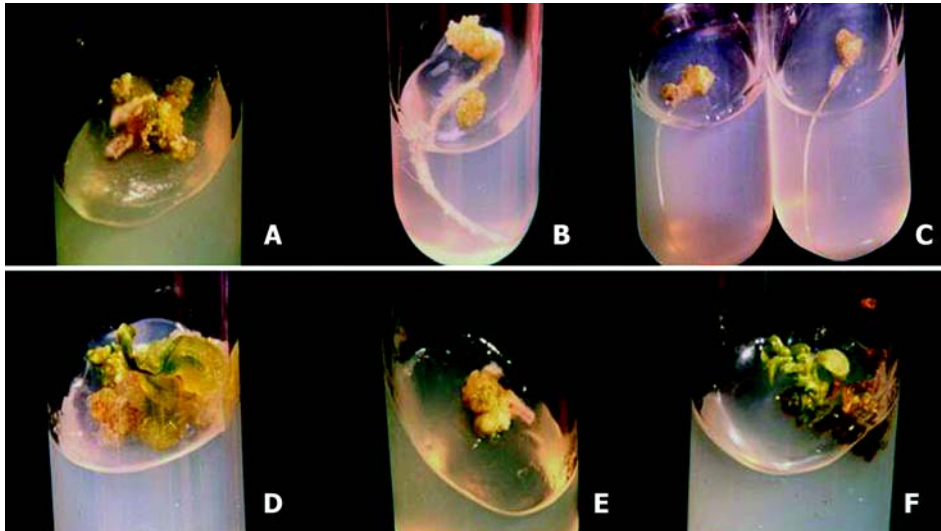


Figure 1: A-Rhizogenic calli of *Helianthus decapetalus* (AC6)  
 B-Thick root formation of *Helianthus annuus* × *Helianthus decapetalus* (AC4)  
 C-Root elongation in *Helianthus decapetalus*  
 D-Leaf differentiation of *Helianthus annuus* × *Helianthus tuberosus* (AR8)  
 E-Pink root growth of *Helianthus annuus* × *Helianthus tuberosus*  
 F-Shoot development of *Helianthus annuus* × *Helianthus tuberosus* (AR8)

The genotypes produced friable to compact calli and similarly the color ranged from light white (Plate A), cream color to light yellow callus (Table 5). The *H. decapetalus* anther callus in some cases produced roots induced from AC6 treatment and in AC4 (Plate B, C) *H. annuus* × *H. tuberosus* produced pink roots (Plate E).

In regeneration media, *H. annuus* produced only friable green callus in most of the treatments except AR8 and AR9 where in friable green embryogenic nodular calli were produced. Later shoot differentiation was observed in AR8. *H. occidentalis*, *H. tuberosus* and *H. annuus* × *H. occidentalis* showed green and embryogenic calli in all the treatments (Table 6). *H. decapetalus* rooted anther callus was transferred to basal MS medium to arrest root growth and for further regeneration. Instead of cessation of root growth, the roots got elongated (plate D). Similarly the friable calli of this species in AR7 treatment produced thick rootlike structure after 15 days of plating in regeneration media.

*H. annuus* × *H. tuberosus* hybrid anther calli showed embryogenesis in AR4 and AR8 treatments. This hybrid showed friable green embryogenic calli. Later the meristematic regions began to elongate only in AR8 medium and leaf differentiation (Plate E) was observed after 30 days of plating and, from that, shoot differentiation occurred (Plate F). On the 60<sup>th</sup> day in regeneration medium AR8, a plantlet was developed with 5-6 leaves and the height of 2.00 cm.

Table 3: Effect of different treatments on anther callus induction in *Helianthus* ssp.

Treatment	<i>H.annuus</i>		<i>H.occidentalis</i>		<i>H.decapetalus</i>		<i>H.tuberosus</i>		<i>H.occidentalis</i>		<i>H.annuus</i> x <i>H.tuberosus</i>	
	P	R	%	P	R	%	P	R	%	P	R	%
AC1	59.00	0.00	0.00	57.00	0.00	0.00	55.00	0.00	60.00	0.00	54.00	0.00
AC2	60.00	58.28	97.13	60.00	52.96	88.26	60.00	51.94	86.57	62.00	50.00	77.80
AC3	60.00	53.00	88.32	60.00	46.69	77.81	60.00	54.54	90.90	60.00	51.28	85.40
AC4	60.00	51.88	86.34	60.00	42.86	71.44	60.00	42.88	71.46	60.00	57.18	95.20
AC5	60.00	55.94	93.23	50.00	50.00	100.00	61.00	58.00	95.11	60.00	52.52	87.50
AC6	60.00	27.41	45.68	60.00	27.64	46.07	60.00	55.39	92.33	60.00	52.81	88.60
AC7	58.00	57.00	98.27	56.00	53.00	94.67	54.00	41.67	77.19	64.00	64.00	100.00
P - Anthers plated, R - Anthers responded, % - Percentage of callus induction												
Source	SEd											
Media	0.861											
Genotype	0.472											
Media x genotype	1.491											
	CD (0.05)											
	1.722											
	0.943											
	2.982											

Table 4: Number of days to callus initiation (CI) and maximum callus (MC) proliferation from anthers of *Helianthus* ssp.

Treatment	Additives (mg l <sup>-1</sup> )														
	Response of callus														
	CH	NAA	2,4-D	BAP	<i>H.annuus</i>		<i>H.occidentalis</i>		<i>H.decapetalus</i>		<i>H.tuberosus</i>		<i>H.annuus</i> x <i>H.tuberosus</i>		
				IC	MC	IC	MC	IC	MC	IC	MC	IC	MC	IC	MC
AC1	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AC2	0	0.1	0.0	0.2	8.0	19.0	8.0	30.0	6.0	24.0	7.0	25.0	10.0	25.0	6.0
AC3	0	2.0	0.0	1.0	9.0	24.0	9.0	29.0	8.0	23.0	9.0	28.0	9.0	26.0	8.0
AC4	250	1.0	0.0	0.5	7.0	25.0	5.0	20.0	5.0	22.0	10.0	27.0	8.0	25.0	7.0
AC5	250	1.0	2.0	0.5	7.0	31.0	7.0	29.0	7.0	26.0	6.0	24.0	6.0	24.0	7.0
AC6	250	2.0	0.1	1.0	6.0	28.0	6.0	25.0	10.0	30.0	8.0	25.0	7.0	25.0	5.0
AC7	500	2.0	0.0	1.0	11.0	32.0	7.0	23.0	9.0	29.0	6.0	24.0	5.0	34.0	8.0
Mean					6.9	22.7	6.0	22.3	6.4	22.0	6.6	21.9	6.4	22.7	5.9
IC - Initiation of callus, MC - Maximum callus proliferation															
Source	SEd														
Media	0.218														
Genotype	0.202														
Media x Genotype	0.535														
	CD (0.05)														
	0.431														
	0.399														
	1.055														
	CD (0.01)														
	0.569														
	0.526														
	1.393														

## DISCUSSION

The anthers at uninucleate stage, which has been optimized by several workers (Mezarroba and Jonard, 1986; Gurel *et al.*, 1991), were inoculated at anther callus induction medium and a good response was evidenced. Callus formation started from the filamentous ends of the anther as observed by Goyal *et al.* (1990). No callus induction was observed in the medium (AC1) wherein no exogenous growth hormone was supplemented. This establishes the fact that endogenous hormones available in the anther explant may not be sufficient to induce callus. Maximum callus induction was observed in the treatment (AC5) that had the auxin 2,4-D in increased amounts (2.0 mg/l) (Punia and Bohorova, 1992; Badizannavar and Kuruvina Shetti, 1998). It may be because of 2,4-D content as it is a powerful suppressant of organogenesis and often induces callus even in the absence of any endogenous cytokinin (Dodds and Roberts, 1985). Next to AC5 treatment, AC2 treatment which contained meager quantities of NAA and BAP (0.1 and 0.2 mg/l, respectively) manifested high callus induction for most of the genotypes and needed a smaller number of days for callus initiation than the other treatments. This indicates the influence of 2,4-D and low auxin and cytokinin contents on early induction of callus (Patil, 1992). However, it was observed that increase in the concentration of auxin and cytokinin (NAA and BAP) does not have significant contribution to callus production (Table 4) (Pugliesi *et al.*, 1993). The treatments in which callus was induced earlier did not give maximum proliferation earlier and *vice versa*. It is argued that nutrients initially available to the anther in a particular medium may not be available throughout the period of culture, in contrast to the late induction medium. Hence for maximum proliferation subculturing at appropriate period depending upon the medium is necessary.

Among the treatments, the genotypes showed differential responsiveness towards callus initiation and proliferation. For instance, in the media composition of AC5 and AC2, *H. decapetalus* had taken less days (7 and 6 days, respectively) for initial callusing than the rest of the genotypes. Bohorova and Atanassov (1990) and Gurel *et al.* (1991) also obtained similar results with their experiments on sunflower.

### **Nature of callus**

The anthers turned brown within 48 to 72 h as observed by Saji and Sujatha (1998). The quality of the callus i.e. its embryogenic nature improved with the concentration of casein hydrolysate in the medium. In AC7, which contained a high amount of CH (500 mg/l), embryogenic nodular calli were produced in most of the genotypes. Conversely, AC2 and AC3 which had not contained CH produced mostly compact calli and no regeneration was observed, contradicting Saji and Sujatha's (1998) report that compact and white calli also produced better regeneration and may be due to genotype influence. *H. tuberosus* anther callus was friable light green

Table 5: Nature of callus derived from anthers of *Helianthus* ssp. through different treatments

Treatment	Additives (mg l <sup>-1</sup> )							Response of callus						
	CH	NAA	2,4-D	BAP	<i>H.annuus</i>	<i>H.occidentalis</i>	<i>H.decapetalus</i>	<i>H.tuberosus</i>	<i>H.occidentalis</i>	<i>H.annuus</i> x <i>H.occidentalis</i>	<i>H.annuus</i> x <i>H.tuberosus</i>			
AC1	0	0.0	0.0	0.0	No response	No response	No response	No response	No response	No response	No response			
AC2	0	0.1	0.0	0.2	Compact light white	Compact light green	Compact light green	Compact light white	Compact light white	Compact light yellow	Compact light yellow			
AC3	0	2.0	0.0	1.0	Friable white	Compact light green	Compact light white	Compact yellow	Friable white	Compact light yellow	Compact light yellow			
AC4	250	1.0	0.0	0.5	Compact light white	Compact light white	Friable light white	Friable light green and rooted	Friable white	Compact light white with thick root	Compact light white with thick root			
AC5	250	1.0	2.0	0.5	Compact light green	Friable light white	Compact light yellow	Dull white	Friable light yellow	Friable light yellow	Friable light yellow			
AC6	250	2.0	0.1	1.0	Friable white	Friable light white	Compact light white and rooted	Friable white	Friable white	Friable white	Friable white			
AC7	500	2.0	0.0	1.0	Light white embryogenic	Compact light white	Friable creamy nodular	Compact light white nodular	Friable green	Compact light white	Compact light white			

and rhizogenic in AC4 treatment (MS + CH 250 mg/l + NAA 1.0 mg/l + BAP 0.5 mg/l). Such root induction might be due to the presence of low cytokinin along with high auxin content (Pugleisi *et al.*, 1993 and Saji and Sujatha, 1998). It is inferred that, for induction of calli, growth regulators are not an essential criterion whereas for obtaining embryogenic calli, optimum dosage of growth regulators is needed (Table 5).

#### Regeneration studies

Most of the genotypes established more embryogenic and regenerating points in the media containing more CH and BAP (AR8 and AR9) than in the rest of the treatments. Hence it was suggested that high dosages of CH and BAP instigated the regeneration potential of the genotypes. But Saji and Sujatha (1998) reported that shoots can be developed only with BAP at 0.5 mg/l.

In AR7 treatment, which contained a low amount of cytokinin, *H.decapetalus* developed root in the medium. This confirms our earlier discussion that a low cytokinin content in the medium promotes root development. However, the same was also observed in the treatment that has more cytokinin (*H.annuus* × *H.tuberosus* in AR9). These contradictory results on root formation could be due to the differences in the ploidy of the tissues under study (Saji and Sujatha, 1998). But even in AR8 and AR9 conditions, the genotype *H.occidentalis* and *H.decapetalus* produced only friable green calli and no embryogenic callus was found. This proves the differential regenerative potential of the genotypes towards regenerating media composition and from this it can be extrapolated that regeneration potential in sunflower is under multigene control with incomplete dominant genes (Paterson and Everett, 1985; Punia and Bohoroava, 1992).

On further subculturing, only *H.annuus* × *H.tuberosus* formed shoot budlike structures and subsequently plantlets (Plate F) in the media AR8 and AR9. The growth regulators thus impose a profound influence on the regeneration of plantlets but are dependent mostly on the genetic makeup of the inbreds (Jeyamary and Jeyabalan, 1997). This was well substantiated by the observation that differentiation of roots was evidenced only for some genotypes in different regeneration media.

In general, kinetin has no specific influence on regeneration frequency of callus. It can only supplement BAP as cytokinin. Mostly, embryogenic calli were differentiated as plantlets directly surpassing the embryoid stage as reported by Gurel *et al.* (1991) whereas Nurhidayah *et al.* (1996) reported that the anther culture of interspecific hybrids in sunflower follows the normal embryogenesis pattern.

Differentiation and morphogenetic responses for rooting were greatly dependent on the concentrations of auxins used. The growth regulator IBA was found to have a greater influence on rooting.

Table 6: Nature of anther callus on different regeneration media in *Helianthus* ssp.

Treatment	Additives (mg l <sup>-1</sup> )			<i>H. annuus</i>	<i>H. occidentalis</i>	<i>H. decapetalus</i>	<i>H. tuberosus</i>	<i>H. annuus</i> × <i>H. occidentalis</i>	
	CH	BAP	Kin					<i>H. annuus</i> × <i>H. tuberosus</i>	<i>H. tuberosus</i> × <i>H. occidentalis</i>
AR1	100	0.2	0	Friable green	Friable green	Friable light yellow	Friable green	Compact green embryogenic	Friable green embryogenic
AR2	100	0.5	0	Friable green	Friable light green	Friable green	Friable green	Compact green embryogenic	Friable green embryogenic
AR3	100	0.5	0.5	Friable light green	Friable green	Friable light yellow	Friable green	Compact green embryogenic	Compact green embryogenic
AR4	250	0.2	0.0	Friable green	Friable green	Friable green	Friable green	Friable light yellow	Friable green embryogenic
AR5	250	0.5	0.0	Friable green	Friable green	Friable green	Friable light yellow	Friable green	Friable light yellow
AR6	250	0.5	0.5	Friable green	Friable green	Friable green	Friable green	Friable light green	Friable green
AR7	500	0.2	0.0	Friable light yellow	Friable light yellow	Thick root	Friable light yellow	Friable light yellow	Friable green embryogenic
AR8	500	0.5	0.0	Friable green proliferated embryogenic with shoot	Friable green proliferated	Friable light green	Friable green embryogenic	Friable green proliferated	Friable green embryogenic proliferated with leaf differentiation
AR9	500	0.5	0.5	Friable green embryogenic nodular	Friable green proliferated	Friable light green	Friable light green embryogenic	Friable light green with shoot differentiation	Nodular friable green proliferated with root

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## REACCIÓN ANDROGENÉTICA DE GIRASOL EN DIFERENTES CULTIVOS

### RESUMEN

La producción de embriones derivados de las microsporas del cultivo de antera, hoy en día representa una técnica ya bien establecida para el aislamiento de las líneas homocigotas en girasol. Nuestro ensayo se ha efectuado con el objetivo de optimizar el contenido de cultivos para *Helianthus* salvaje e híbridos interespecies. Las anteras de microsporas mononucleares se han cultivado en la base MS basal con diferentes combinaciones de hormonas. La inducción del callo ha sido rápida y abundante con la presencia de 2,4-D y bajo contenido de citoquinina y auxina. El exceso de citoquinina y auxina no tenía importancia para la inducción de callo. El potencial para la regeneración se aumentó en la cantidad aumentada/óptima del producto de hidrólisis de caseína y bencil amino purina. Cinetina no tenía influencia concreta en la frecuencia de regeneración de callo. El genotipo ha tenido una influencia significativa en la capacidad de inducción de callo y en formación de plántulas. La combinación *H. annuus* × *H. tuberosus* reaccionó bien y produjo plántulas en la base con el óptimo cultivo. Por androgénesis de interespecies del híbrido *H. annuus* × *H. tuberosus* se formaron plántulas que sobrepasan la fase de embrión.

## RÉACTION ANDROGÉNÉTIQUE DU TOURNESOL À DIFFÉRENTES CULTURES

### RÉSUMÉ

La production d'embryons issus de microspores de culture d'anthers est aujourd'hui une technique bien établie pour l'isolation de lignes homozygotes dans le tournesol. Nous avons fait cette expérience dans le but d'optimiser la composition de la culture pour l'*Helianthus* sauvage et les hybrides interspecies. Les anthers de microspores à noyau unique ont été cultivées dans un bouillon de culture MS avec différentes combinaisons d'hormones. L'induction de callus a été rapide et abondante en présence de 2,4-D et le contenu de cytoquinine et d'auxine, faible. Le surplus de cytoquinine et d'auxine n'a pas eu d'importance pour l'induction de callus. Le potentiel de régénération a été accru avec la quantité augmentée/optimale du produit de l'hydrolyse de la caséine et de l'amine purine de benzyle. La quinétine n'a pas eu d'influence concrète sur la fréquence de régénération du callus. Le génotype a eu une influence importante sur la capacité d'induction du callus et la formation des plantules. La combinaison *H. annuus* × *H. tuberosus* a bien réagi et a produit des plantules dans le bouillon de culture optimale. L'hybride interspecies *H. annuus* × *H. tuberosus* a formé par androgénèse des plants qui ont dépassé le stade d'embryon.

