Embryo rescue in Sunflower (*Helianthus annuus L.*), a simple method to obtain more generations per year.

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Abstract
Embryo culture of 3 Sunflower lines was used to study the establishement of dormancy by the analysis of germination responses on different culture media of immature embryos.
The results indicate: i) embryos of 10 days old are able to germinate even in distilled water, ii) differences among genotypes become consistent during the establishment of dormancy, iii) successful generations cicling may be performed in climatic chamber with high density of plants.

Introduction
Embryo development can be divided essentially into two stages: the first, during which from the zygote through extensive mitotic divisions, the bipolar plan of the embryo is realized and the second of maturation when with cell expansion takes place the deposition of reserves in the storage tissues. Generally dormancy is established at the end of the first phase in order to maintain the embryo in a development mode until it is fully formed and has accumulated sufficient reserves to permit successful germination and subsequent seedling growth (Kermode A.R. 1990 ; Corbineau F. et al 1990; Gay C. et al. 1991)
In this context embryo rescue may be used either to overcome the sterility of some interspecific crosses due to abnormality of embryo development by the supply with the media of a compatible environment, or to prevent the establishment of dormancy allowing a precocious germination.
In this report we analyse the second possibility in order to shorten the biological cycle of sunflower and to have more generations per year.

Materials and methods
Experiments were conducted on 1994-1995. The genetic materials were 3 inbred lines (maintainer and male-sterile forms), selected at the genetics section of plant biology Department of Pisa.

For identification purposes the following codes are used: line A1 = BL207A, line A2 = GM2113A, line A3 = AC2221A.
Plants were grown in climatic chamber at 25° C, 60% relative humidity in 18/6 light/dark cycles (light from fluorescent tubes Sylvania day light F36 W/56 with the intensity of 250 mol / m.s). At flowering impollinations were made collecting pollen from the maintainer plants and transporting it on the male-sterile counterpart.
The developing achenes were collected at two days intervals from fertilization to physiological maturity. Achenes were gently decoated then surface sterilized for 1 min. in 70% (v/v) ethanol and for 20 min. in 2,8% (v/v) sodium hypochlorite solution. After 3 rinses in sterile distilled water, embryos were posed on the medium in Petri plates (200x15 mm.) sealed with parafilm and incubated at 25 °C in 16/8 light/dark cycles (light from fluorescent tubes Sylvania Day Light F36 W/56 with the intensity of 250 mol / m.s).
The basal medium of Muraschige & Skoog (MS)(1962), with 3% of sucrose, 0,8% of bactoagar (oxoid Ltd, England) was supplemented or not with Indole-3-acetic acid (IAA) and N6-benzylaminopurine (BAP), prior to autoclaving at 120°C for 15 min..
Media and the relative hormonal concentrations were:
1) DW - = Distilled water
2) MS1 = MS basal medium without hormones
3) MS2 = MS + 0,4 mg/l BAP + 0,1 mg/l IAA
4) MS3 = MS + 0,1 mg/l BAP + 0,4 mg/l IAA

After 7 days of incubation, germination percentage was calculated considering germinated the seeds that had expanded cotyledons and elongated the radical axis.
Statistical unit was the Petri plate with 50 embryos, 5 replicates for each treatment and for each genotype were used.
The generation cycling was performed only for embryos germinated on distilled water (DW medium)
40 germinated embryos for each line and for each stage of development were transplanted in jiffy pots (diam 6 cm) filled with a mixture of soil and sand plus an initial dose of complete fertilizer, the pots were placed in trays filled with vermiculite and grown till maturity in the growth chamber with the same climatic conditions of mother plants, water restoration was performed empirically when plants showed symptoms of water deficit.

Results

In table 1 are reported the percentages of germination in relation to the embryo age and the utilized media.
The comparative analysis of the table indicates :

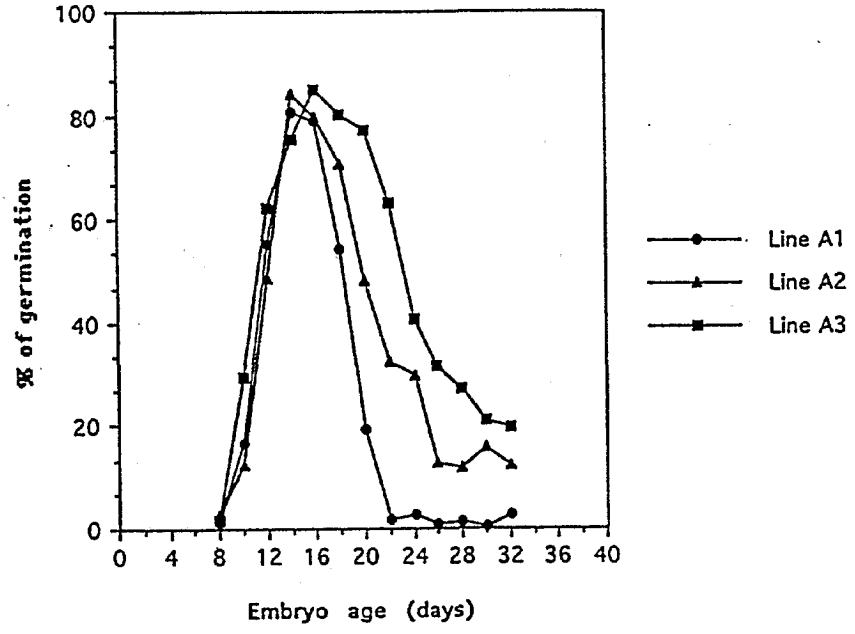
i) Only during the first phases of development, embryo is sensitive to the hormonal supply, particularly medium MS2 is able to induce germination when embryo is 6 days old, the effect is more evident in line A1 and line A3.
ii) After 10 days of development embryo is fully formed and able to germinate even in distilled water, from that point the effects of the different germination media are in fact ineffective.

Tab 1 - germination percentage (mean and standard error) of the line A1, A2, A3 in relation to the embryo age and the utilized media. (for codes identification see materials and methods)

	Days	D.W.	MS0	MS1	MS2
Line A1					
	6	-	0,30+0,1	2,50+0,6	10,9+2,4
	8	1,3+0,4	0,6+0,2	6,8+1,7	8,9+1,5
	10	16,7+3,6	20,1+2,8	17,8+3,5	23,4+2,8
	12	55,4+4,7	52,9+3,8	48,9+4,9	51,4+4,6
	14	80,7+5,9	84,5+4,8	79,3+6,7	82,3+3,8
	16	78,8+3,2	80,9+4,1	76,2+4,5	80,5+6,1
	18	54,3+2,4	49,7+3,5	57,2+3,8	60,8+6,5
	20	19,2+4,8	22,3+3,8	19,5+2,8	25,4+4,2
	22	1,8+0,4	4,3+0,9	3,8+0,5	5,3+0,8
	24	2,5+0,4	0,8+0,2	1,5+0,4	0,5+0,2
	26	0,8+0,2	1,2+0,3	0,7+0,1	2,2+0,5
	28	1,2+0,4	2,1+0,1	1,3+04	0,9+0,3
	30	0,5+0,2	1,1+0,2	0,9+0,4	0,5+0,1
Line A2					
	6	-	0,1+0,0	4,2+0,3	8,5+1,1
	8	2,5+0,3	1,7+0,1	3,8+0,1	12,9+1,8
	10	12,3+1,8	15,6+2,8	18,5+3,1	20,3+2,8
	12	48,8+4,5	52,4+4,9	46,8+5,1	54,3+4,2
	14	84,2+5,9	79,5+6,3	82,7+4,1	87,1+7,2
	16	79,8+4,5	82,4+5,9	80,5+7,8	78,8+4,9
	18	70,8+8,1	72,1+7,3	78,5+3,9	72,8+5,7
	20	48,3+4,7	42,1+6,4	50,2+7,1	44,3+2,8
	22	32,5+2,8	28,7+4,7	37,4+5,8	30,2+3,1
	24	29,8+3,4	30,5+4,6	31,5+5,1	30,4+4,7
	26	12,8+3,4	16,1+2,9	15,3+3,9	11,8+4,3
	28	11,9+2,1	15,2+3,7	20,8+4,1	16,3+2,8
	30	15,8+4,5	14,8+2,9	12,6+4,7	15,9+3,2
Line A3					
	6	-	1,2+0,0	6,5+0,2	11,8+1,7
	8	1,9+0,3	2,0+0,2	9,9+1,5	15,2+2,3
	10	29,2+3,1	22,1+4,2	30,1+2,0	28,9+3,5
	12	62,3+3,9	57,6+3,8	60,4+4,0	58,6+2,9
	14	75,4+5,1	80,3+5,3	80,2+6,1	75,2+4,7
	16	84,9+3,1	81,2+5,7	78,2+3,2	83,7+4,8
	18	80,2+4,0	82,7+7,4	75,1+5,5	80,1+5,0
	20	77,2+5,1	70,5+4,1	80,2+4,3	73,5+3,0
	22	63,2+3,1	60,2+4,7	68,9+3,4	61,7+2,5
	24	40,9+5,6	50,0+6,2	45,2+4,2	50,1+4,8
	26	31,4+4,2	33,2+5,1	40,3+4,7	38,4+3,9
	28	27,2+3,8	25,9+2,8	26,7+2,7	30,8+4,0
	30	20,9+2,4	19,2+2,0	22,3+3,0	18,7+4,5

In fig. 1 are reported the germination percentages on distilled water in relation to the embryo age of the 3 lines analysed.
The maximum level of germination is reached when embryos are 14-16 days old, after that point dormancy goes to be established and germination declines rapidly. It is interesting to notice that the differences among lines become consistent only during this phase of embryo development: embryos of 22 days old are completely dormant in line A1, while embryos of line A2 and line A3 reach a minimum of germination (15%, 20%) when they are 32 days old.

Fig.1-Germination percentage in distilled water



In tables 2 are reported means and standard errors of plant height, capitulum diameter, seed number per head and duration of phenological phases for the 3 lines analysed.
It is evident that plants coming from immature embryos and grown in climatic chamber show a reduction in the initial growth rate resulting in shorter and herlier plants compared to the field control. Capitulum diameter was reduced to 2, 4 cm., while the seed set was enough to ensure a successful generation cycling of 4, 5 generations per year.

Tab.2-Means and standard errors for some phenological and agronomic traits in 3 sunflower lines grown in climatic chamber (cl.ch) and in the field.

	V	R1-R5	R5-R9	H.f	C.diam	N.seed
Line A1						
cl.ch	48,2+2	31,2+3	12	21,5+2	3,1+0,2	25,7+7
field	54,1+3	30,4+1	35,3+4	120,2+4	21,3+2,1	853+72
Line A2						
cl.ch.	42,5+3	27,4+2	12	17,4+1	2,4+0,4	18,3+6
field	48,9+3	26,7+1	32,8+5	89,4+7	18,2+2	545+42
Line A3						
cl.ch.	39,4+4	28,4+2	12	25,9+7	3,0+0,4	12,1+4
field	45,2+2	29,1+1	40,6+9	145+5	19,2+3	652+94

Note : -

V	Duration of vegetative phase (from emergence to bud differentiation)
R1-R5	Duration of reproductive phase (from bud differentiation to anthesis)
R5-R9	Duration of maturation phase (from flowering to physiol. maturity)
H.f	Height at flowering
C. diam	Capitulum diameter
N.seed	Number of seeds per head

Discussion

The technique of embryo rescue using agarized media with different hormonal concentrations has been successfully used to overcome sterility of some interspecific sunflower hybrids (Chandler J.M. and Beard B.H., 1983;), the same technique with agarized media has been used also for rapid generation cycling by various authors (Alissa A. et al. 1986; McCann et al. 1988; Gopalkrishnan K. 1993). Our results indicate that in cultivated sunflower the germination of immature embryos is not influenced by the supply of auxins and cytokinins and the same germination rate may be obtained even in distilled water just when embryos are 10 days old. Using this technique we have been able to obtain 4, 5 generations per year. This is of particular interest because this technique avoids the tedious sterilization methods facilitating breeding work especially when a large amount of genotypes has to be manipulated. Furthermore, according to Pistolesi et al. (1986) growing plants in climatic chamber under reduced soil and nutrient availability it is able to reduce plant size allowing the growth of about 200 plants per square meter.

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