Progress on the Sunflower Doubled Haploid Project

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Introduction

Haploidy refers to the condition of any organism, tissue or cell having the chromosome constitution of the normal gametes of the species involved.

Haploid plants can be obtained either spontaneously or induced through androgenesis, gynogenesis or wild hybridization.

Doubling the haploid chromosome number will produce a doubled haploid (DH).

Androgenesis consists of production of plants from anthers or microspores cultured in vitro.

Doubled haploids have important applications in plant genetic and breeding research.

In sunflowers, some methods such as anther culture, microspore culture and irradiation of pollen grains have been tried by researchers to produce DHs.

Anther culture is one of the most used methods. Cultivated sunflowers have proven to be very recalcitrant in anther culture, especially for shoot regeneration.

Until now, none of the techniques used have been successfully applied to Doubled Haploid breeding programs of sunflowers.

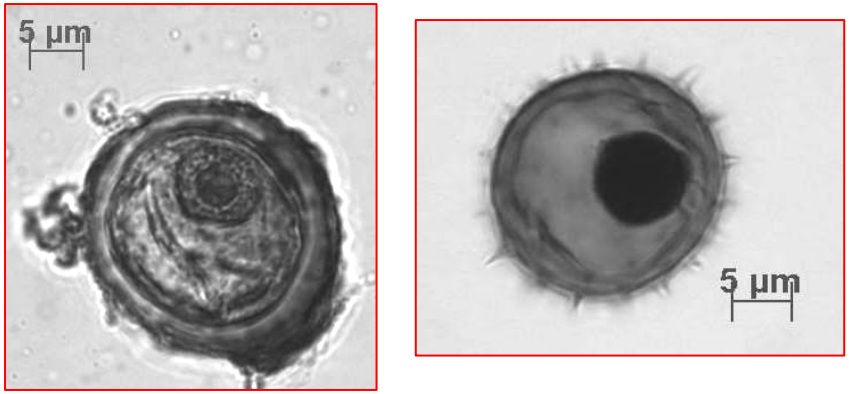
In October 2010, the Sunflower Doubled Haploid Project was initiated by the USDA. This report gives the progress of this project for 2011.

Anther culture

Basic process

A) inbred lines and amphiploid hybrids were tested.

B) Anthers with microspores at the late uninucleate stage were used for culturing, which are light yellow-white in appearance.



microspores at the late uninucleate stage

C) 30% bleach solution for 10 min was chosen for explant sterilization.

D) Anthers were excised under a stereo binocular microscope and plated in 100×15 mm Petri dishes containing 25 ml of medium for culture.

E) Anthers were cultured at 35°C in dark for 12d, then cultured at 25°C in dark.

Embryonic callus or Embryo-Like-Structures (ELS) induction

Anther culture of inbred lines

HA89, HA410, RHA280, RHA274, Peredovik, RHA801, Seneca and Hopi Dye.

Our experiment used L9 (3^4) orthogonal design.

Table 1 L9 (3^4) orthogonal design for factors and levels on embryonic callus induction

Experim-ent and Medium code	Factors			
	1- Genotype	2 – Sucrose (g/l)	3- Phytohormone (mg/l)	4- Organic addition (per litre medium)
E1	RHA274	120	BAP0.5+NAA0.5	No addition
E2	RHA274	90	BAP1.0+NAA0.5	CH 500mg
E3	RHA274	30	BAP0.5+2,4-D 0.5	Coconut water 100ml
E4	HA89	120	BAP1.0+NAA0.5	Coconut water 100ml
E5	HA89	90	BAP0.5+2,4-D 0.5	No addition
E6	HA89	30	BAP0.5+NAA0.5	CH 500mg
E7	Peredovik	120	BAP0.5+2,4-D 0.5	CH 500mg
E8	Peredovik	90	BAP0.5+NAA0.5	Coconut water 100ml
E9	Peredovik	30	BAP1.0+NAA0.5	No addition

Notes:

1) Basic medium: MS (macro +micro-elements) +Agar 7g/l + Morel and Wetmore's medium vitamins + a series of amino acids (AA) .

2) BAP---6-benzylaminopurine, 2,4-D---2,4-dichlorophenoxyacetic acid, NAA---naphthlcetic acid.

3) Every experiment consisted of 3 replications (petri dishes) , each with 150~ 200 anthers.

Table 2 Induction results of embryonic callus or ELS

Experiment.	Material	No. of anthers cultured	No. of callus	Rate of callus (%)
E1	RHA274	515	3	0.61±0.65
E2	RHA274	525	46	8.99±3.52
E3	RHA274	650	1323	205.77±111.44
E4	HA89	575	0	0
E5	HA89	595	29	5.00±5.00
E6	HA89	520	0	0
E7	Peredovik	585	396	67.87±20.37
E8	Peredovik	395	7	1.78±0.54
E9	Peredovik	405	3	0.74±0.49

Table 3 ANOVA results of embryonic callus or ELS induction

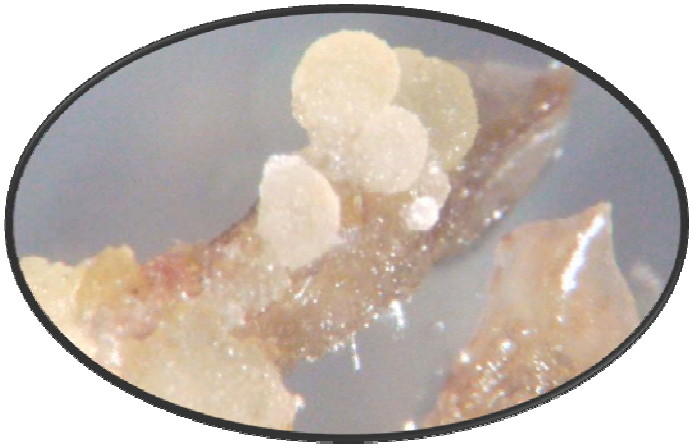
Source	*DF*	*SS*	Mean Square	*F* Value	*Pr > F*
Replication	2	2724.38	1362.19	0.91	0.4245
Genotype	2	22649.31	11324.65	7.58	0.0059*
Sucrose	2	22102.91	11051.46	7.39	0.0064*
Phytohormone	2	50622.66	25311.33	16.93	0.0002*
Organic addition	2	17624.44	8812.22	5.90	0.0139*

* Significance at *P* = 0.05 level.

Table 4 T Grouping of embryonic callus or ELS induction

T Grouping	Mean	N	Experiment
A	205.77	3	E3
B	67.87	3	E7
B	8.99	3	E2
B	5.00	3	E5
B	1.78	2	E8
B	0.74	2	E9
B	0.61	3	E1
B	0	3	E6
B	0	3	E4

Note: Means with the same letter are not significantly different.



ELS produced in one anther

a cluster of ELS distributed along the whole anther

many ELS produced in all five anthers of one flower

ELS development in anthers of RHA274 on medium E3

In our experiment, we discovered a genotype-medium combination, E3, which had the highest induction of ELS.

The effectiveness of this medium was confirmed using more inbred lines and interspecific amphiploids.

Table 5 Induction results of embryonic callus or ELS

Medium	Material	No. of anthers cultured	No. of callus or ELS	rate of callus (%)
E3	HA89	350	10	2.86±0.12
E3	HA410	575	109	18.96±1.43
E3	RHA274	530	909	171.51±20.67
E3	Peredovik	370	50	13.51±18.61
E3	RHA801	755	427	56.56±3.58
E3	Seneca	360	92	25.56±3.14
E3	Hopi Dye	315	175	55.55±0.30
E3	G08/2260	200	48	24.00±40.96
E3	G08/2263	470	95	20.21±6.79
E3	G08/2266	305	27	8.85±3.14

Amount of callus induction changed among genotypes, but improved on the whole.

HA410---
anther base
swelled
then
callused

RHA280
---
anther
wall
callused

Responses of anthers of different genotypes on induction medium

Anther culture of amphiploid hybrids

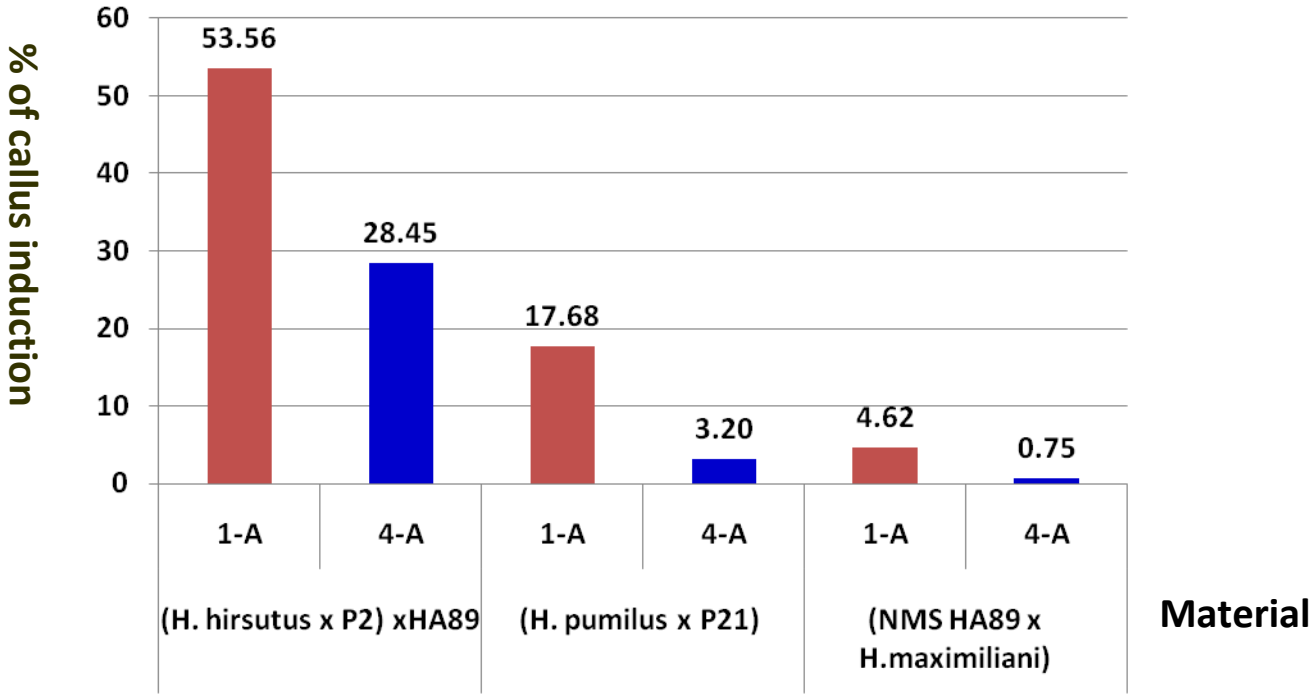


Figure 1 Anther callus induction of amphiploid hybrids

1-A: anthers with microspores at the late uninucleate stage

4-A: anthers with microspores at or after the binucleate stage

Effects of chemical inducers on anther culture

Three chemical inducers were tested on anther cultures:

- **2-hydroxynicotinic acid (2-HNA),**
- **2-(*p*-chlorophenoxy)-2-methylpropionic acid (PCIB),**
- **24-epibrassinolide (EBR).**

Eight inbred lines were used : HA89, HA410, RHA280, RHA274, Peredovik, RHA801, Seneca, and Hopi Dye.

For chemical inducer 2-HNA, sunflower heads with 10-cm long stems were precultured in 2-HNA solutions at 32°C in the dark. Four combinations of 2-HNA concentration and time were used.

Pretreatment of sunflower heads by 2-HNA

Time \ 2-HNA / Pretreatment / Code	100mg/l	50mg/l
48 h	a	b
72 h	e	f
0	CK	

Table 6 Anther callus induction results by 2-HNA pretreatment

Material And pretreatment code	No. of anthers cultured	No. of callus	Rate of callus (%)
HA89-a	495	12	2.42
HA89-b	430	14	3.26
HA89-e	565	14	2.48
HA89-f	290	1	0.34
HA89-CK	728	113	15.52
HA410-a	360	20	5.56
HA410-b	340	22	6.47
HA410-e	390	2	3.08
HA410-f	425	18	4.24
HA410-CK	419	16	3.82
RHA280-a	170	3	1.76
RHA280-b	190	23	12.11
RHA280-e	470	32	6.81
RHA280-f	0	0	0
RHA280-CK	630	8	1.26
RHA274-a	395	51	12.91
RHA274-b	325	64	19.69
RHA274-e	615	55	8.94
RHA274-f	660	0	0
RHA274-CK	1130	138	12.21

Material And pretreatment code	No. of anthers cultured	No. of callus	Rate of callus (%)
Peredovik-a	725	56	7.72
Peredovik-b	540	83	15.37
Peredovik-e	380	3	0.79
Peredovik-f	250	1	0.45
Peredovik-CK	652	62	9.51
RHA801-a	545	27	4.95
RHA801-b	195	1	0.51
RHA801-e	810	14	1.73
RHA801-f	365		0
RHA801			5.15
Senec			.44
Senec			.51
Senec		7	3.5
Seneca-f	410	4	0.98
Seneca-CK	1300	183	14.08
Hopi Dye-a	130	8	6.15
Hopi Dye-b	350	4	1.14
Hopi Dye-e	595	30	5.04
Hopi Dye-f	380	48	12.63
Hopi Dye-CK	660	126	19.09

Callus induction did not increase.

Table 7 Anther callus induction results by adding PCIB and EBR

Material and Medium	No. of anther cultured	No. of callus	Rate of callus (%)
HA89-A1	103	0	0
HA89-A3	104	1	0.96
HA410-A1	112	0	0.00
HA410-A3	115	0	0.00
RHA280-A1	46	0	0.00
RHA280-A3	58	0	0.00
RHA274-A1	88	1	1.14
RHA274-A3	81	1	1.23

Material and Medium	No. of anther cultured	No. of callus	Rate of callus (%)
Peredovik - A1	128	3	2.34
Peredovik - A3	116	1	0.86
RHA801-A1	48	0	0.00
RHA801-A3			0.00
Seneca			
Seneca			
Hopi D A1			3.26
Hopi Dye - A3		0	0.00

Neither 10 µM PCIB nor 0.1 µM EBR had a positive effect on improving induction of anther calli.

Note: A1--- PCIB 10µM; A3--- EBR 0.1µM.

Plant regeneration of embryonic callus and ELS

Plant regeneration media

Media code	Ingredients
S1	MS + BAP 0.5mg/l + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l
S2	MS + BAP 0.5mg/l + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l+ coconut water 100ml/l
S3	MS + BAP 1.0 mg/l + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l
S4	MS + BAP 1.0 mg/l + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l+ coconut water 100ml/l
S5	MS + BAP 0.5mg/l+ Sucrose 20g/l +Agar 7g/l+ coconut liquid 100ml/l
S6	MS + BAP 0.25mg/l + Sucrose 20g/l +Agar 7g/l
S7	MS + Sucrose 20g/l +Agar 7g/l
S8	MS + BAP 0.5 mg/l + NAA0.1mg/l+ Sucrose 20g/l +Agar 7g/l
S9	MS + BAP 0.5mg/l + IBA0.1mg/l+ Sucrose 30g/l +Agar 7g/l
S10	MS + BAP 0.5 mg/l + NAA0.1mg/l+ Sucrose 20g/l +Agar 7g/l+ coconut water 100ml/l

IBA---Indole-3-butyric acid.

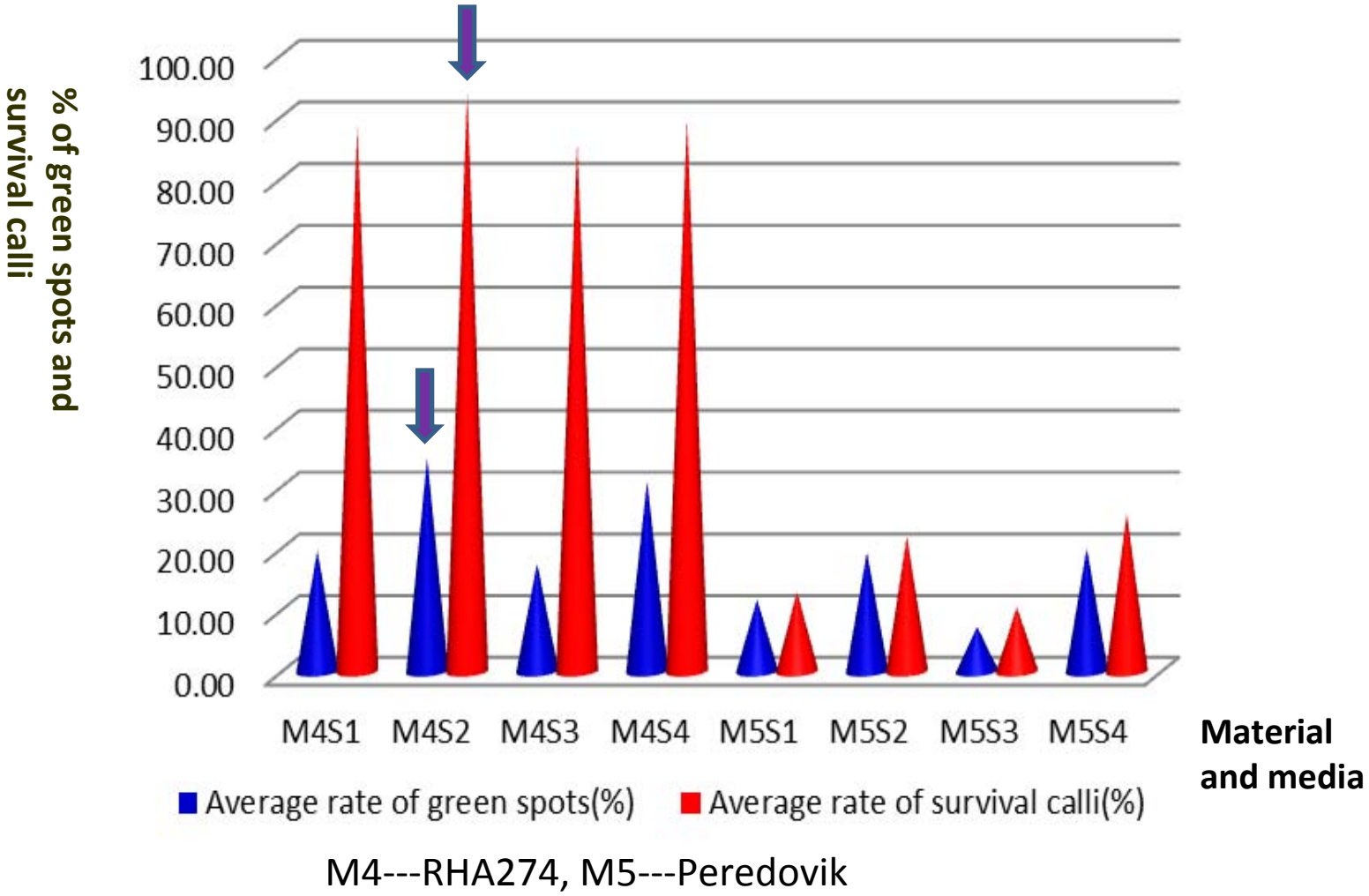


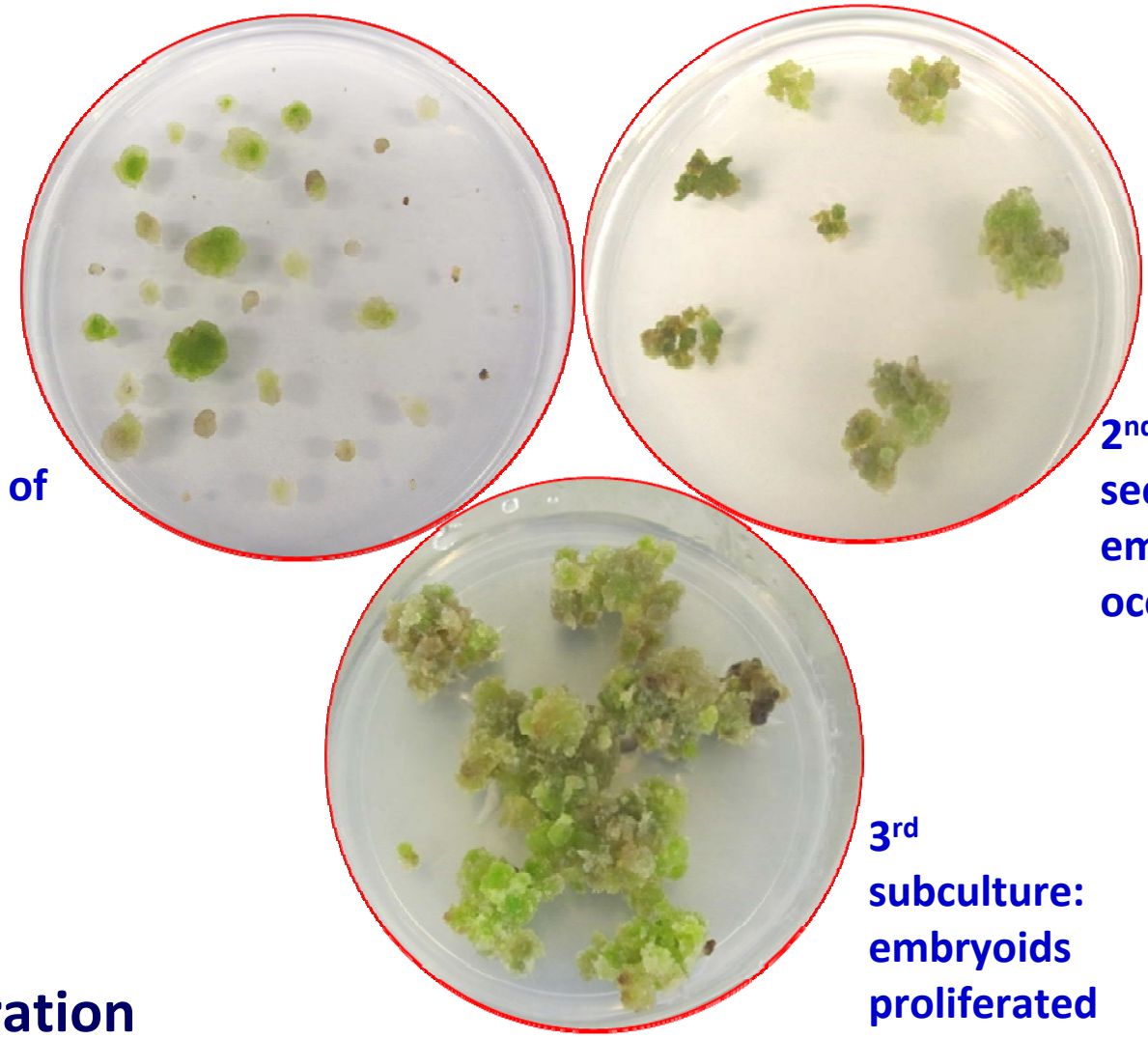
Figure 2 The first sublture of anther callus: regeneration results of two inbred lines

Medium S2 produced a higher green spot conversion rate (about 35%) than the others during the first subculture for plant regeneration.

Many subculture steps are normally required to produce shoots from callus or ELS.

During the subculture, some genotypes such as RHA274 and amphiploids of *H. pumilus* ×P21, produced embryoids from callus or ELS through secondary embryogenesis, then proliferated.

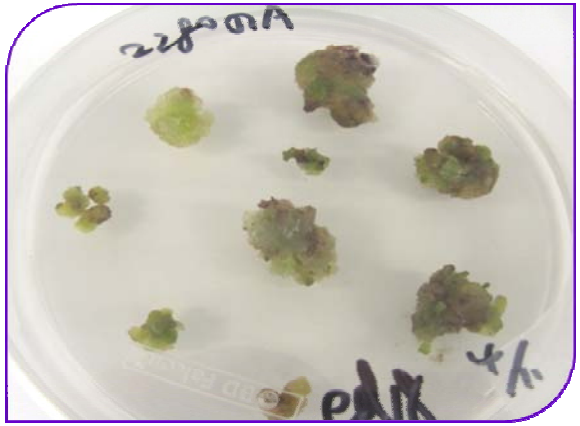
Plant regeneration process of RHA274



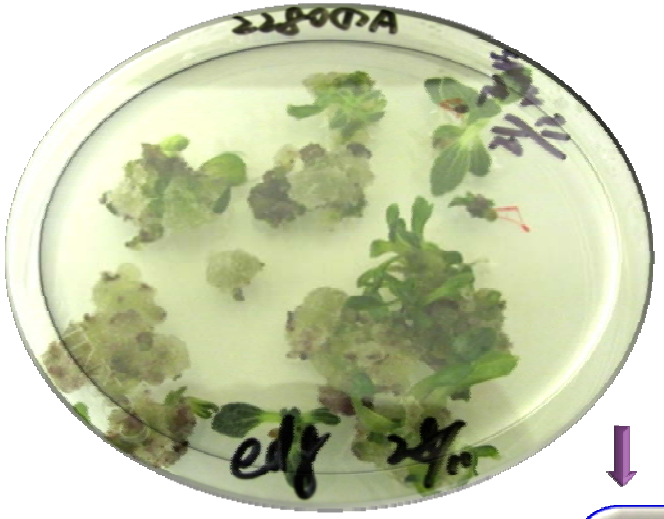
1st subculture of ELS on S2 medium

2nd subculture: secondary embryogenesis occured

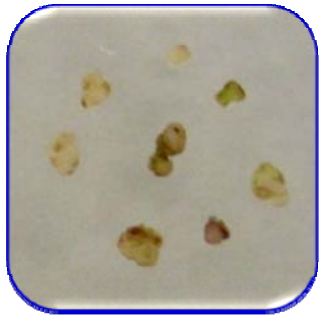
3rd subculture: embryoids proliferated



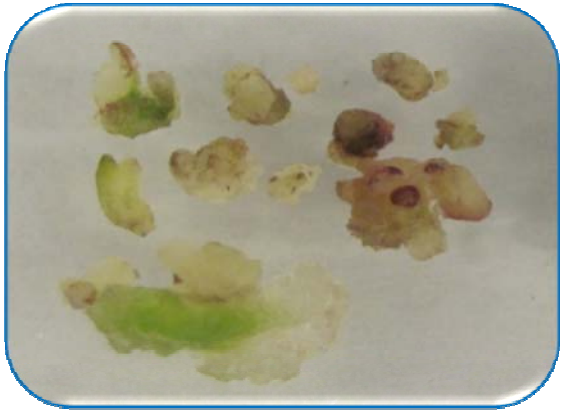
Embryoid on regeneration medium



Embryoid germination



Embryoid at different developmental stages



Small shoot elongation

Normal plantlets

Abnormal plantlet

Plant regeneration process of amphiploid *H. pumilus* ×P21

For plant regeneration, the inbred lines did not produce shoots from callus or ELS, unlike the amphiploids.

Amphiploid *H. pumilus* ×P21 had the most plant regeneration of the experimental materials used. More than 100 plantlets have been produced in rooting media and soils to date.

***NMS HA89×H. maximiliani*, amphiploid**

***H. pumilus*×P21, amphiploid**

Shoot elongation on hormone-free medium before transferring to rooting medium

Rooting of the regenerated plantlets

Rooting media

Media code	Ingredients
r1	½ MS + NAA1.0mg/l+ Sucrose 20g/l +Agar 7g/l
r2	½ MS + IBA 1.0mg/l+ Sucrose 20g/l +Agar 7g/l
r3	½ MS + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l
r4	½ MS + IBA 0.5 mg/l+ Sucrose 20g/l +Agar 7g/l
r1a	½ MS + NAA1.0mg/l+ Sucrose 20g/l +Agar 7g/l+ AC 0.5g/l
r2a	½ MS + IBA 1.0mg/l+ Sucrose 20g/l +Agar 7g/l+ AC 0.5g/l
r3a	½ MS + NAA0.5mg/l+ Sucrose 20g/l +Agar 7g/l+ AC 0.5g/l
r4a	½ MS + IBA 0.5 mg/l+ Sucrose 20g/l +Agar 7g/l+ AC 0.5g/l

AC----activated charcoal

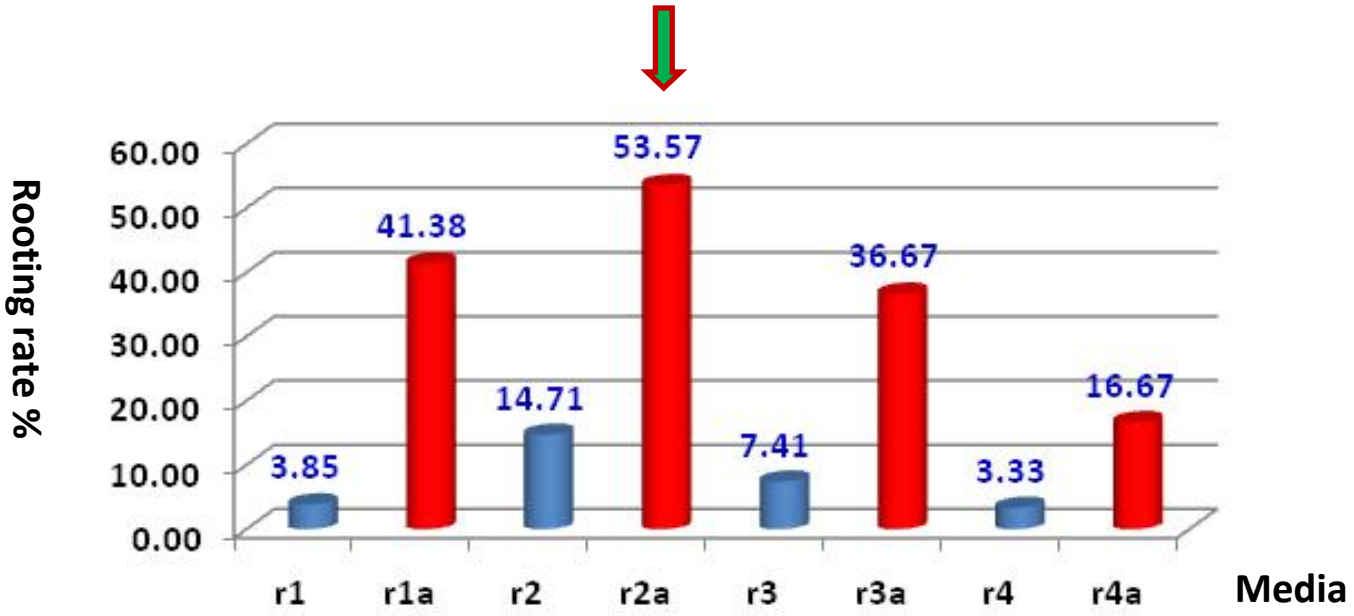


Figure 3 Rooting rate of amphiploid *H. pumilus* ×P21 shoots

r2a medium has the highest rooting rate among all the experimental media. Adding Activated Charcoal to the rooting medium is helpful for shoot rooting.

+ AC **-AC**

Shoots in rooting medium

Plantlets transplanted to peat pellets

Our results produced plantlets from anther culture of three amphiploid hybrids, NMS HA89 ×*H. maximiliani* , *H. pumilus* ×P21, and (*H.Hirsutus*×P21) × HA89.

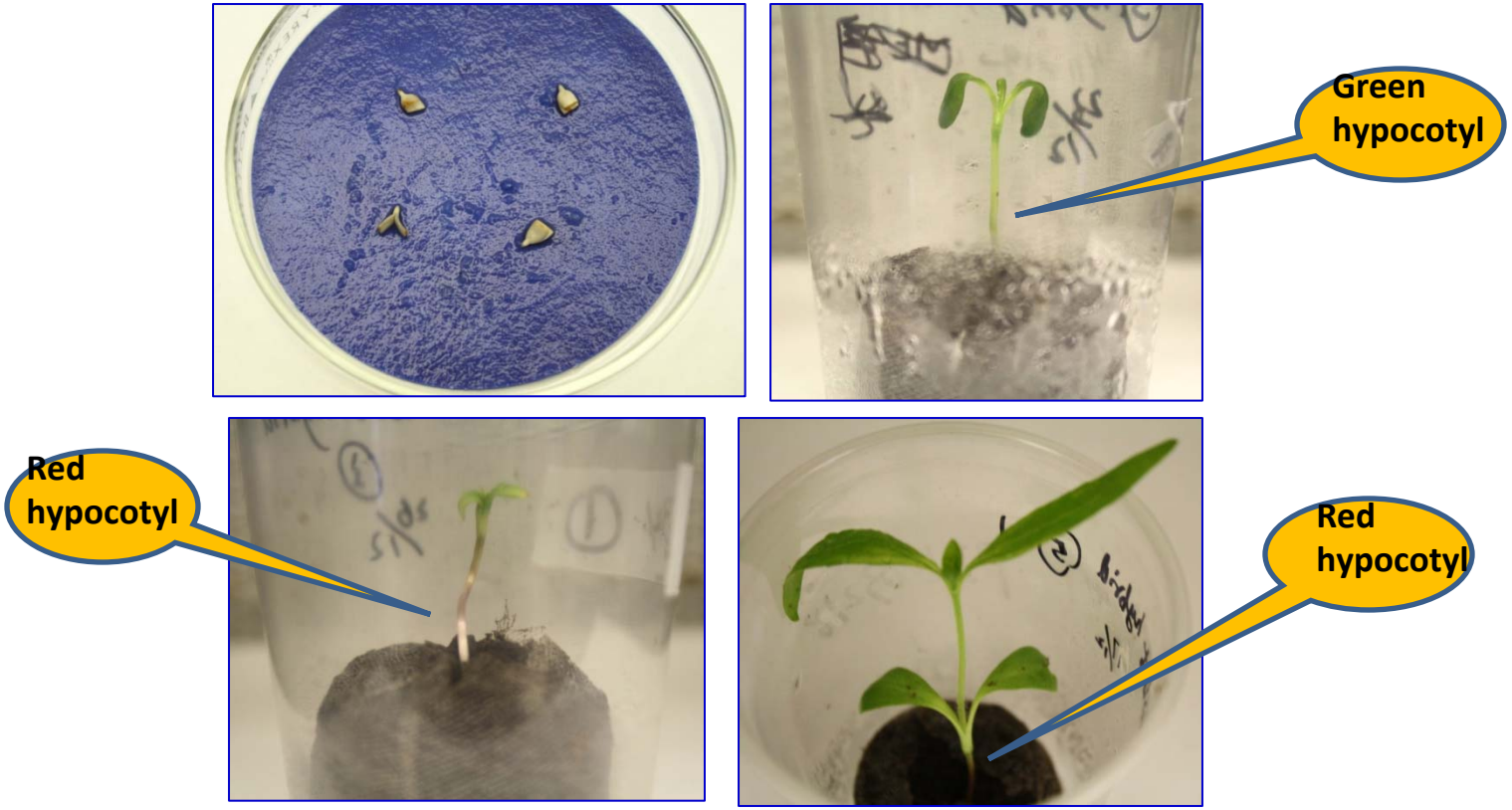
Some of the plantlets rooted successfully.

Chromosome numbers of these plantlets will be determined.

Foreign pollen inducers

Interspecific hybridizations between cultivated sunflowers and *H. tuberosus* were made. Twenty one hybrid seeds were obtained.

Female parent (♀)	Male parent (♂)	No. of heads pollinated	No. of hybrid seeds harvested	Percent seed set (%)
(CMS HA412HO ×HA467) F_1	*H. tuberosus* (West Fargo)	4	10	0.357
(CMS HA412HO ×HA467) F_1	*H. tuberosus* (Kindred South)	2	10	0.714
(CMS HA412HO ×HA467) F_1	*H. tuberosus* (Moorhead)	3	1	0.048



The hybrid seeds are germinating in culture.

These three seedlings all derived from the combination:

(CMS HA412HO ×HA467) F_1 × *H. tuberosus* (Kindred South).

Future plans

- **Transition from anther culture to microspore culture.**

- **Continue "Foreign pollen inducer" work. Detect haploids by counting chromosome of the hybrid seedlings.**

- **Pursue "Induced mutation" work. Dr. Brent Hulke plans to testcross M2 progenies to determine whether or not any individual mutation line leads to haploid progeny.**

- **Establish true haploid lines, develop doubled haploid lines through chromosome doubling.**

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