

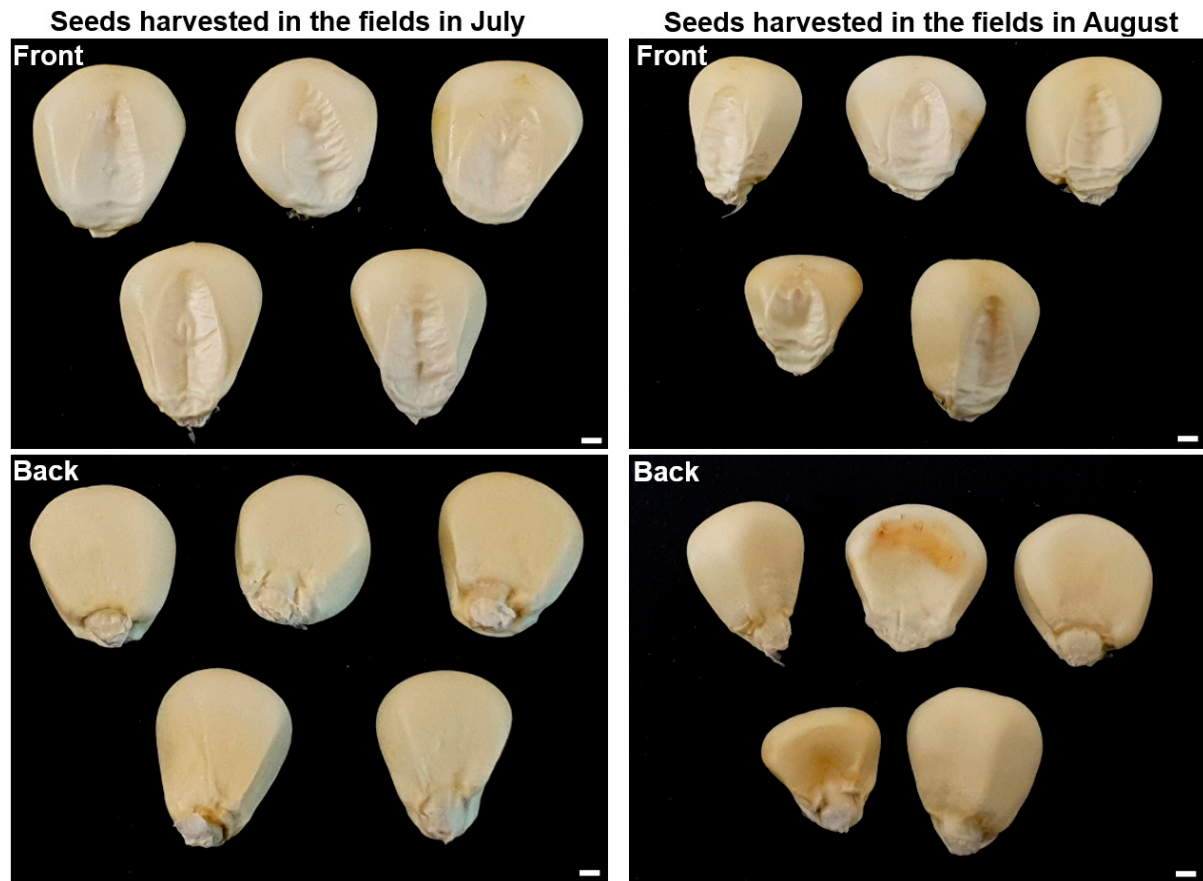
**Supplementary materials**

**A New Protocol to Mitigate Damage to Germination Caused by Black  
Layers in Maize (*Zea mays* L.) Seeds**

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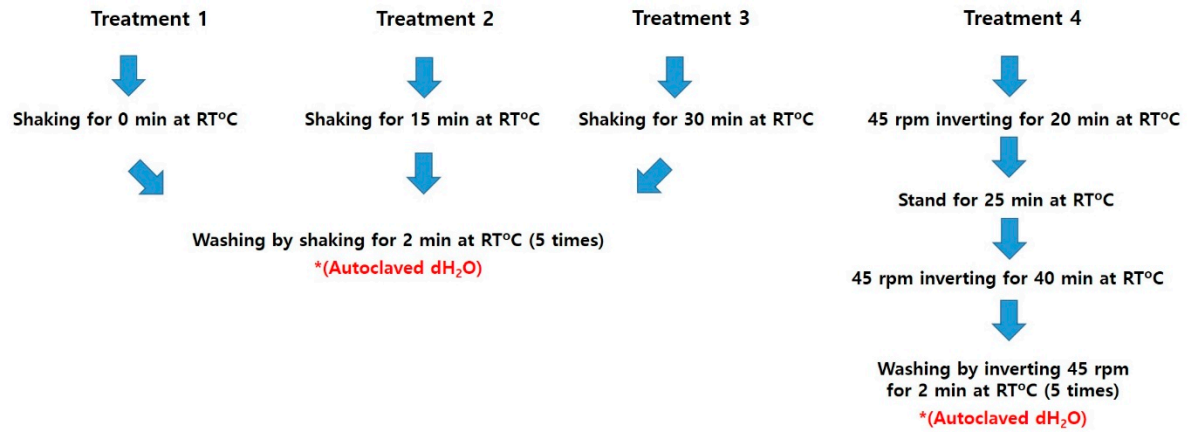
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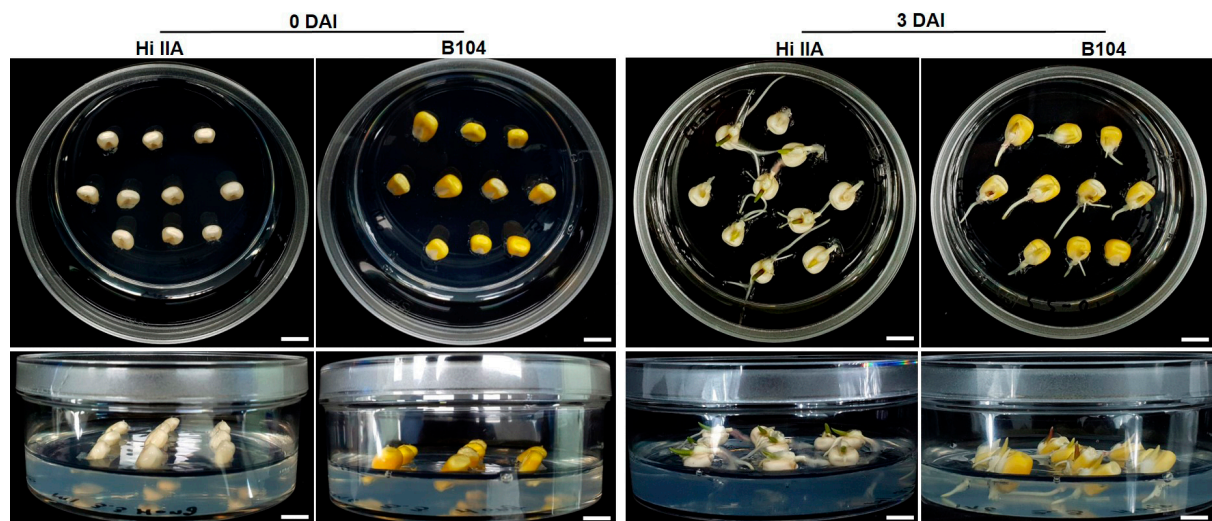
**Figure S1.** Phenotypes of maize Hi IIA seeds harvested from fields in July and August. Seeds harvested in August were more contaminated by microorganisms than seeds harvested in July. Seeds that were severely contaminated were removed, and the remaining seeds were mixed and used for research. Bars = 1 mm.

### Maize seed sterilization methods

- 20 grains (35ml sterilized dH<sub>2</sub>O – control )
- 20 grains (35ml 100% commercial bleach + 35 µl Tween20)



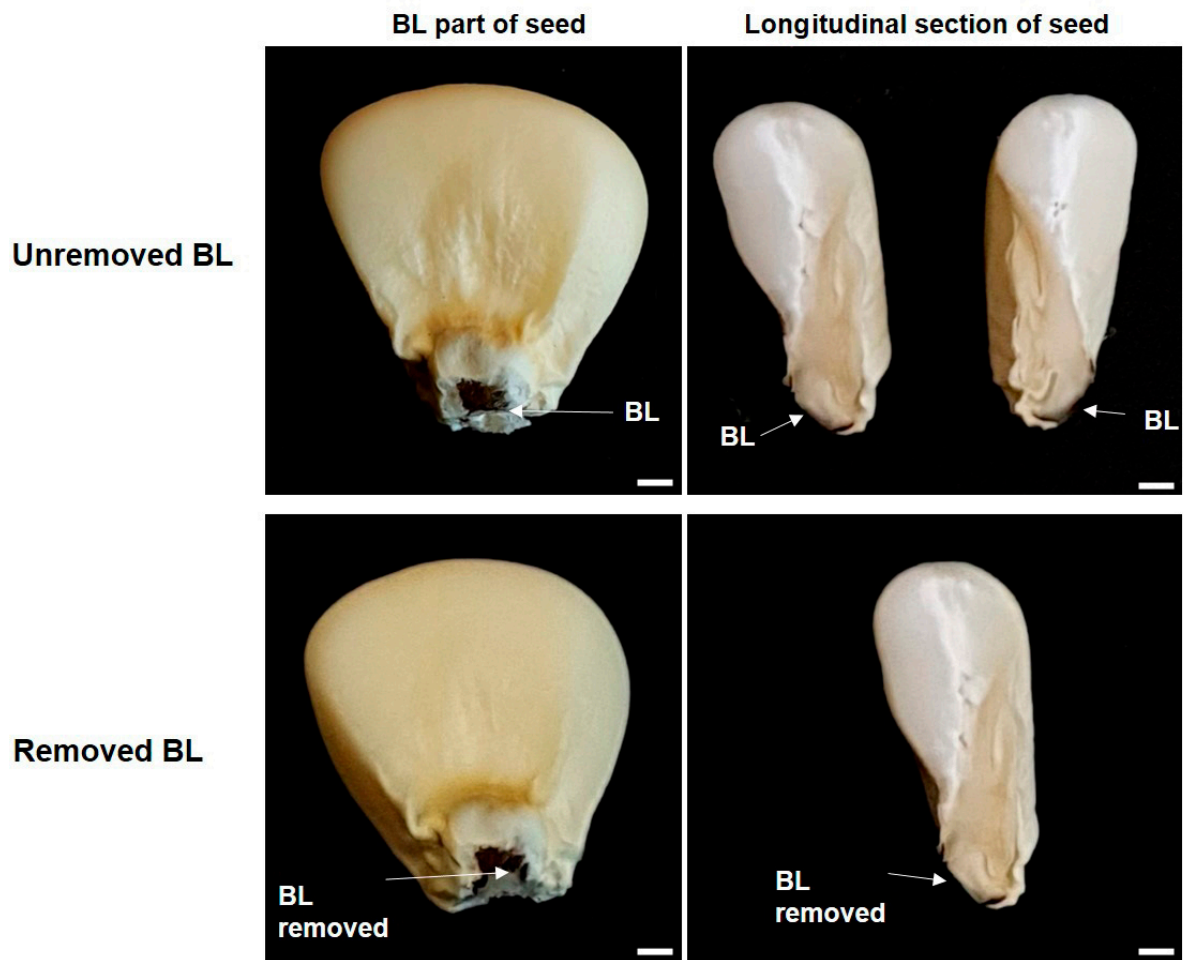
**Figure S2.** The applied methods of maize seed sterilization.



**Figure S3.** Placement and growth of sterilized maize seeds on the MS medium. The maize seeds was placed to 40~45 angles by touching the medium, the germination was confirmed 3 days after imbibition. DAI, days after imbibition. Bars = 1 cm.



**Figure S4.** Germination of maize Hi IIA seeds after exposure to sterilization treatment. (A) Seed germination and seedling development. (B) Longitudinal cut of seed during germination. Bars = 1 cm.

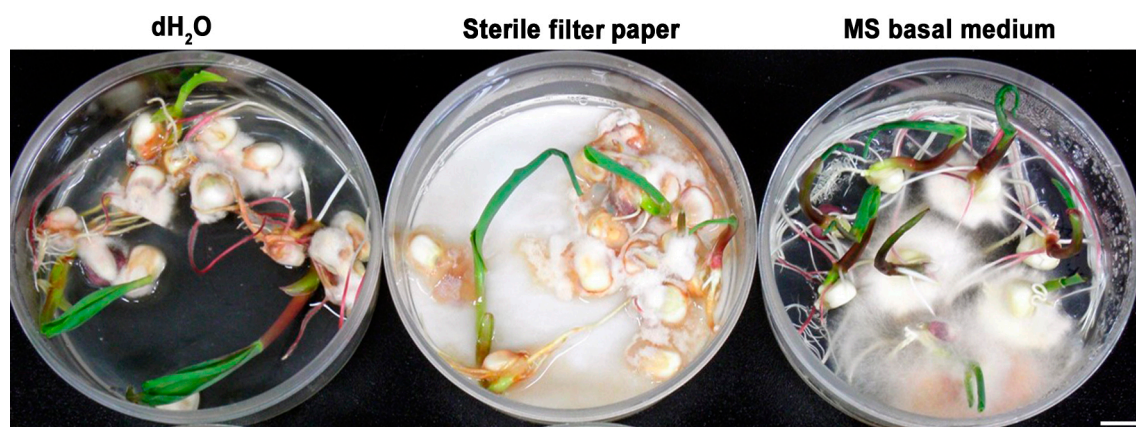


**Figure S5.** Location and removal area of the black layer in maize Hi IIA seeds. In order not to damage the inside of the seed, only a part ( $1\sim4\text{ mm}^2$ ) of the black layer was removed. BL, the black layer of maize seed. Bars = 1 mm.





**Figure S6.** Standard flat with 50 hole-pots filled with potting mix for planting maize Hi IIA seeds. Fifteen-day-old germinated seedlings in greenhouse. Bar = 1 cm.

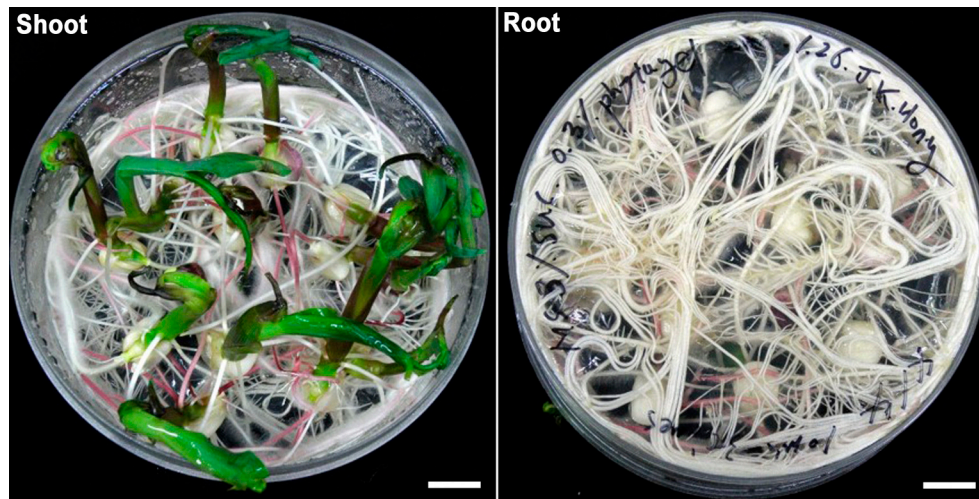


**Figure S7.** Microbial growth on maize Hi IIA seeds sterilized by the standard method. The sterilized seeds were placed on the sterile dH<sub>2</sub>O, sterile filter paper, and MS basal medium and the growth of microorganisms was observed for 7 days. Bar = 1 cm.





**Figure S8.** Maize Hi IIA seedlings grown for 4 days in the medium after exposure to sterilization treatment 4 were transplanted into pots and grown in growth chamber for 7 days. Bar = 5 cm.



**Figure S9.** Maize Hi IIA seedlings growth after exposure to sterilization treatment 4. Macroscopic images showing microbial growth on the basal medium for 10 days after seed sterilization. Bars = 1 cm.



**Figure S10.** After sterilization maize seeds with optimized sterilization method, greenhouse growth.

**Table S1.** Germination of maize seeds in pots.

Variety	Germination						
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	Mean (%)	Std.Dev	Std.Err
Hi IIA	1000	313	308	321	31.40	0.66	0.38
Hi IIA× Hi IIB	1000	485	387	399	42.37	5.35	3.09
A188	1000	659	618	639	63.87	2.05	1.18
H99	1000	634	614	624	62.40	1.00	0.58
B104	1000	884	863	891	87.93	1.46	0.84
B73	1000	946	921	951	93.93	1.61	0.93
B98	1000	963	954	971	96.27	0.85	0.49
HW3	1000	707	699	721	70.90	1.11	0.64
KS140	1000	948	933	920	93.37	1.40	0.81
KS141	1000	983	990	964	97.90	1.35	0.78
Hi IIA(♂)×B73(♀)	300	122	119	127	40.89	1.35	0.78
B73(♂)×Hi IIA(♀)	300	103	93	101	33.00	1.76	1.02

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, Std.Dev; standard deviation, Std.Err; standard error.



**Table S2.** Contamination and germination of maize Hi IIA seeds after exposure sterilization treatment 1.

Variety	Germination							Contamination			
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)
Hi IIA	300	294	290	292	97.33	0.67	0.38	300	300	300	100
Hi IIA× Hi IIB	300	298	297	294	98.78	0.69	0.40	300	300	300	100
A188	300	298	293	295	98.44	0.84	0.48	300	300	300	100
H99	300	297	296	294	98.56	0.51	0.29	300	300	300	100
B104	300	298	295	297	98.89	0.51	0.29	300	300	300	100
B73	300	300	298	299	99.67	0.33	0.19	300	300	300	100
B98	300	300	298	299	99.67	0.33	0.19	300	300	300	100
HW3	300	296	294	295	98.33	0.33	0.19	300	300	300	100
KS140	300	299	298	291	98.67	1.45	0.84	300	300	300	100
KS141	300	300	299	299	99.78	0.19	0.11	300	300	300	100
Hi IIA(♂)×B73(♀)	150	149	144	149	98.22	1.92	1.11	150	150	150	100
B73(♂)×Hi IIA(♀)	150	147	149	144	97.78	1.68	0.97	150	150	150	100

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G.Mean; germination rate mean (%), Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C. Mean; contamination rate mean.

**Table S3.** Contamination and germination of maize Hi IIA seeds after exposure sterilization treatment 2.

Variety	Germination							Contamination					
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Hi IIA	300	294	290	292	97.33	0.67	0.38	122	128	127	41.89	1.07	0.62
Hi IIA×Hi IIB	300	298	297	294	98.78	0.69	0.40	101	106	97	33.78	1.50	0.87
A188	300	298	293	295	98.44	0.84	0.48	89	99	74	29.11	4.19	2.42
H99	300	297	296	294	98.56	0.51	0.29	80	78	83	26.78	0.84	0.48
B104	300	298	295	297	98.89	0.51	0.29	78	71	76	25.00	1.20	0.69
B73	300	300	298	299	99.67	0.33	0.19	76	77	78	25.67	0.33	0.19
B98	300	300	298	299	99.67	0.33	0.19	82	80	81	27.00	0.33	0.19
HW3	300	296	294	295	98.33	0.33	0.19	77	74	70	24.56	1.17	0.68
KS140	300	299	291	298	98.67	1.45	0.84	92	88	84	29.33	1.33	0.77
KS141	300	295	296	294	98.33	0.33	0.11	98	101	84	31.44	3.02	1.75
Hi IIA(♂)×B73(♀)	150	150	149	147	99.11	1.02	0.59	52	56	53	35.78	1.39	0.80
B73(♂)×Hi IIA(♀)	150	150	148	148	99.11	0.77	0.44	54	53	56	36.11	0.84	0.48

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C. Mean; contamination rate mean.



**Table S4.** Contamination and germination of maize Hi IIA seeds after exposure sterilization treatment 3.

Variety	Germination							Contamination					
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Hi IIA	300	296	294	295	98.33	0.33	0.19	59	55	61	19.44	1.02	0.59
Hi IIA×Hi IIB	300	298	294	296	98.67	0.67	0.38	61	60	54	19.44	1.26	0.73
A188	300	298	300	297	99.44	0.51	0.29	56	50	52	17.56	1.02	0.59
H99	300	296	299	297	99.11	0.51	0.29	57	56	57	18.89	0.19	0.11
B104	300	298	299	293	98.89	1.07	0.62	53	48	66	18.56	3.10	1.79
B73	300	300	297	299	99.56	0.51	0.29	58	65	51	19.33	2.33	1.35
B98	300	300	294	298	99.11	1.02	0.59	61	70	54	20.56	2.67	1.54
HW3	300	297	295	299	99.00	0.67	0.38	59	50	62	19.00	2.08	1.20
KS140	300	299	300	291	98.89	1.64	0.95	60	55	57	19.11	0.84	0.48
KS141	300	300	297	299	99.56	0.51	0.29	63	66	61	21.11	0.84	0.48
Hi IIA(♂)×B73(♀)	150	149	145	147	98.00	1.33	0.77	26	30	33	19.78	2.34	1.35
B73(♂)×Hi IIA(♀)	150	148	149	145	98.22	1.39	0.80	29	27	31	19.33	1.33	0.77

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.

**Table S5.** Contamination and germination of maize Hi IIA seeds after exposure sterilization treatment 4.

Variety	Germination							Contamination					
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Hi IIA	300	296	300	297	99.22	0.69	0.40	38	28	33	11.00	1.67	0.96
Hi IIA×Hi IIB	300	298	299	295	99.11	0.69	0.40	39	34	30	11.44	1.50	0.87
A188	300	298	294	295	98.56	0.69	0.40	37	32	36	11.67	0.88	0.51
H99	300	296	299	300	99.44	0.69	0.40	38	31	33	11.33	1.20	0.69
B104	300	298	289	299	98.44	1.84	1.06	37	27	39	11.44	2.14	1.24
B73	300	300	298	295	99.22	0.84	0.48	35	31	30	10.67	0.88	0.51
B98	300	300	299	297	99.56	0.51	0.29	39	29	40	12.00	2.03	1.17
HW3	300	297	299	299	99.44	0.38	0.22	41	29	30	11.11	2.22	1.28
KS140	300	299	300	294	99.22	1.07	0.62	40	34	31	11.67	1.53	0.88
KS141	300	300	299	294	99.22	1.07	0.62	37	33	27	10.78	1.68	0.97
Hi IIA(♂)×B73(♀)	150	149	144	147	97.78	1.68	0.97	15	24	23	13.78	3.29	1.90
B73(♂)×Hi IIA(♀)	150	148	142	148	97.33	2.31	1.33	19	20	21	13.33	0.67	0.38

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.

**Table S6.** Contamination and germination of maize Hi IIA seeds during different sterilization treatments with removed black layer.

Treatment s	Germination							Contamination					
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Treatment 1	300	291	294	291	97.33	0.58	0.33	300	300	300	100.00	0.00	0.00
Treatment 2	300	294	299	298	99.00	0.88	0.51	106	99	101	34.00	1.20	0.69
Treatment 3	300	295	297	296	98.67	0.33	0.19	34	27	24	9.44	1.71	0.99
Treatment 4	300	296	299	299	99.33	0.58	0.33	3	1	0	0.44	0.51	0.29

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.

**Table S7.** Effects of inverting RPM change during maize seed sterilization process 4 with removed black layer of maize Hi IIA seeds.

		Germination							Contamination					
	Conditions	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
RPM	25	300	297	291	299	98.56	1.39	0.80	52	48	49	16.56	0.69	0.40
	45	300	294	296	297	98.56	0.51	0.29	2	0	0	0.22	0.38	0.22
	65	300	298	297	298	99.22	0.19	0.11	37	36	41	12.67	0.88	0.51

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.

**Table S8.** Effects of the number of seeds during maize seed sterilization 4 with removed black layer of maize Hi IIA seeds.

Conditions		Germination							Contamination					
		Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Number of seeds	10	300	298	300	289	98.56	1.95	1.13	2	0	0	0.22	0.38	0.22
	20	300	294	298	299	99.00	0.88	0.51	1	1	0	0.22	0.19	0.11
	30	300	293	299	291	98.11	1.39	0.80	32	29	31	10.22	0.51	0.29

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; ; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.

**Table S9.** Contamination and germination of seeds after exposure sterilization treatment 4 with removed black layer of 11 varieties seeds.

Variety	Germination							Contamination					
	Grains <sup>a</sup>	1th-E	2th-E	3th-E	G. Mean (%)	Std.Dev	Std.Err	1th-C	2th-C	3th-C	C. Mean (%)	Std.Dev	Std.Err
Hi IIA×Hi IIB	300	298	297	296	99.00	0.33	0.19	2	1	1	0.44	0.19	0.11
A188	300	298	294	297	98.78	0.69	0.40	2	0	2	0.44	0.38	0.22
H99	300	296	299	291	98.44	1.35	0.78	2	1	1	0.44	0.19	0.11
B104	300	298	294	297	98.78	0.69	0.40	0	0	0	0.00	0.00	0.00
B73	300	300	299	299	99.78	0.19	0.11	0	0	0	0.00	0.00	0.00
B98	300	300	299	299	99.78	0.19	0.11	0	0	0	0.00	0.00	0.00
HW3	300	297	291	294	98.00	1.00	0.58	0	0	0	0.00	0.00	0.00
KS140	300	299	297	299	99.44	0.38	0.22	2	1	1	0.44	0.19	0.11
KS141	300	300	300	300	100.00	0.00	0.00	2	1	0	0.33	0.33	0.19
Hi IIA(♂) × B73(♀)	150	150	148	149	99.33	0.67	0.38	1	1	0	0.44	0.38	0.22
B73(♂) × Hi IIA(♀)	150	150	150	150	100.00	0.00	0.00	1	0	1	0.44	0.38	0.22

<sup>a</sup> The amount of seeds used in an experiment.

E; germinated seeds, G. Mean; germination rate mean, Std.Dev; standard deviation, Std.Err; standard error, C; contaminated seeds, C.R; contamination rate (%), C. Mean; contamination rate mean.



**Table S10.** Transformation efficiency according to the sterilization method of Hi IIA maize seeds.

Treatment	Experimental year	No. of seeds	No. of transplant / rate (%)	Pollinated ears / pollination rate (%)	No. of immature embryo infected (A)	No. of Bialaphos resistant callus (B)	Transformation Efficiency (B/Ax100, %)
Sterilization treatment 2	18	600	256 / 42.6	174 / 68	293	6	2.05
	19	1,200	464 / 38.6	325 / 70	4,114	127	3.09
	20	1,000	364 / 36.4	186 / 51	2,054	106	4.23
	Total : 18~20	2,800	1,084 / 38.7	685 / 63.2	6,911	239	3.46
Sterilization treatment 4 with black layer removed.	21	500	464 / 92.8	264 / 57	2,343	149	6.36
	22	450	424 / 94.2	140 / 33	2,092	358	17.11
	23	250	230 / 92	75 / 32.6	1,385	ongoing	ongoing
	Total : 21~23	1,200	1,118 / 93.2	464 / 42.8	4,435 <sup>a</sup>	507 <sup>b</sup>	11.43 <sup>c</sup>

<sup>a</sup> Total immature embryo used in the experiment for 2 years ('21 ~'22).

<sup>b</sup> The sum of Bialaphos resistance callus analyzed over a tow-year period ('21 ~'22).

<sup>c</sup> Average value of transformation efficiency for two years ('21 ~'22).