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Genotypes of high competence for somatic embryogenesis and plant regeneration in soybean *Glycine max*

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Summary. Soybean somatic embryos were induced from cultured immature embryos in the presence of a high-level concentration of Naphthalenacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenetic capacity was strongly influenced by genotypes of explants. Induced somatic embryos could be sorted into normal or abnormal types according to the morphological shapes of cotyledons and hypocotyls. Somatic embryos were transferred sequentially to three different media until germination. Germination capacity from the somatic embryos was also influenced by the genotype. Following germination, these regenerated plantlets were transferred to soil in the greenhouse and were stably matured to set seeds.

Key words: Soybean – Somatic embryo – Plant regeneration – Genotype

Introduction

Somatic embryogenesis of soybean was first reported by Christianson et al. (1983). In several other studies somatic embryos were obtained directly, or via callus, from immature embryos (Lippmann and Lippmann 1984; Li et al. 1985; Ranch et al. 1985; Lazzeri et al. 1985; Barwale et al. 1986). A high concentration of auxin, mainly 2,4-D, NAA, IAA, picloram, etc., was tested for embryo induction.

Ranch et al. (1985) tested genotype difference for somatic embryogenesis in 14 genotypes. They also checked the correlation of embryogenic competence to their maturity group, but there was no correlation between them. Barwale et al. (1986) showed that genotype differences such as maturity groups, seed coat color and shoot-forming capacity at the cotyledonaly node did not influence plant regeneration. Frequency of shoot formation ("germination" from somatic embryos) was most restrictive for plant recovery from somatic embryos. Thus, the present study was undertaken to screen the genotypic differences of competence in both somatic embryogenesis and subsequent shoot formation in soybean varieties. In this paper, we report genotypes of high capacity in plant regeneration.

Materials and methods

Seeds of 26 soybean varieties were supplied by Dr. K. Harada, NIAR, Japan (shown in Table 2). They were grown in the field and applied to the tissue culture.

Three culture media used in embryogenesis were the modified medium of Lazzeri et al. (1985). Another four culture media, used for somatic embryo development and plant regeneration, were composed by modification and combination of B5 (Gamborg et al. 1968) and MS (Murashige and Skoog 1962). They are given in Table 1.

Induction of somatic embryos from immature embryos was undertaken according to the procedure of Lazzeri et al. (1985). Immature embryos were previously removed from embryonic axis, and the remaining pair of cotyledons were inoculated on 10 ml of solid culture medium in a test tube. The culture media were MS inorganics and B5 organics (MSB) containing 3% sucrose, growth regulators, pH adjusted to 5.8 prior to autocleave, solidified with gelrite or agar. A total of 50 immature embryos per variety were applied to culture in 26 varieties. Twenty immature embryos were inoculated to both media containing 2 mg/l 2,4-D or 10 mg/l NAA. Another ten immature embryos were inoculated on the same medium, but containing both 10 mg/l NAA and 0.1 mg/l BA (Benzyladenin). Cultures were incubated under continuous dim light at 22 °C for 6 weeks.

At 6 weeks after culture initiation, numbers of induced somatic embryos were counted as either normal types or abnormal types, according to the visual observation on the cotyledons and hypocotyls shapes (see "Results" and "Discus-

Table 1. Culture media components used in this study (mg/l)

Elements	Embryogenesis			Bud and root induction	Embryo development	Embryo germination	Plant development
Major salts	MS	MS	MS	B5 or MS[a]	B5 or MS[a]	MS[a]	MS[a]
Minor salts	MS	MS	MS	B5 or MS	B5 or MS	MS	MS
Vitamins	B5	B5	B5	B5	B5	B5	B5
Sucrose	30,000	30,000	30,000	10,000	10,000	10,000	10,000
NAA	–	10	10	–	–	0.1	–
2,4-D	2	–	–	–	–	–	–
IBA	–	–	–	0.5	–	–	–
BA	–	–	0.1	0.2	–	0.001	–
Gelrite	–	1,800	1,800	4,000	–	2,000	2,000
Agar	8,000	–	–	–	–	–	–
pH	5.8	5.8	5.8	6.0	6.0	6.0	6.0

[a] Half of MS

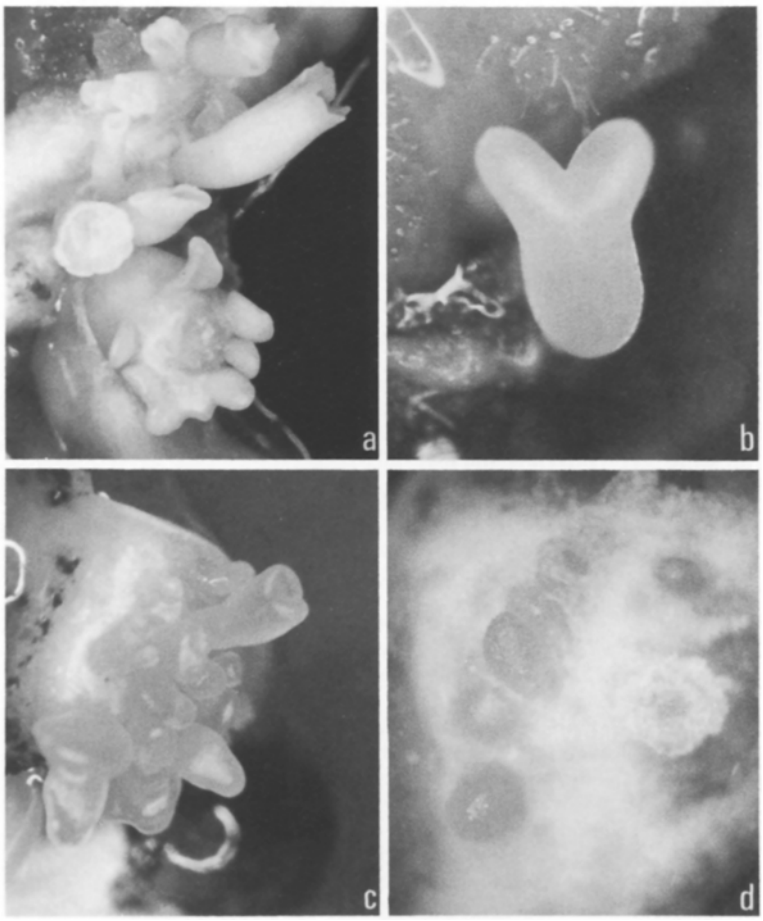

Fig. 1a–d. Shapes of somatic embryos induced from immature embryos of soybean varieties. **a** Various shaped somatic embryos of "Akishirome" induced by 2 mg/l 2,4-D. **b** Somatic embryo of "Akasaya 1" in normal morphology induced by 10 mg/l NAA. **c** Somatic embryos of "Okuhara-daizu" induced by 10 mg/l NAA. **d** Embryoid of "*G. gracilis* T135-589" induced by 2 mg/l 2,4-D; this embryoid became callus after subculture

sion"). All normal types and a portion of abnormal types were collected and applied to maintenance through the three steps of sequential subcultures. First, they were cultured on either B5 or ½ MSB (half strength of major elements) medium containing 1% sucrose, 0.2 mg/l BA, 0.5 mg/l IBA and 0.4% gelrite under continuous dim light at 22 °C for 2 weeks for bud and root induction (Fig. 3a). They were then transferred to B5 or ½ MSB liquid medium containing 1% sucrose in test tubes and cultured at 2 rpm under 19 h light (2000 Lux) for 2 weeks for rapid development (Fig. 3b). Finally, they were cultured on ½ MSB medium solidified with 0.2% gelrite containing 0.1 mg/l NAA, 0.001 mg/l BA and 1% sucrose, cultured under 19 h light (5000 Lux) at 26 °C, and subcultured every 4 weeks until germination.

Once an embryo germinated to form stem and leaves, it was transferred to ½ MSB (1% sucrose, lacking growth hor-

Table 2. Number of embryogenic culture (EC), total somatic embryos (TE) and relative callus growth (Cs, Cr, Cc)

Growth regulators	2 mg/l 2,4-D			10 mg/l NAA			10 mg/l NAA & 0.1 mg/l BA		
No. of explants	20 explants			20 explants			10 explants		
Genotype	EC	TE	Cs[a]	EC	TE	Cr[a]	EC	TE	Cc[a]
G. gracilis	9	42	+	9	25	–	1	2	+ +
G. gracilis T34	19	133	+	19	81	–	0	0	+ +
G. gracilis T135-589	4	14	+ +	9	20	–	0	0[b]	+ + +
G. gracilis T135-590	8	27	+	1	3	–	0	0	+ + +
Masshokutou (kou 502)	15	50	+	12	54	–	0	0[b]	+ +
Masshokutou (kou 503)	18	72	– +	16	77	–	0	0[b]	+
Okuhara-daizu	13	47	+ +	19	179	–	0	0	+ +
Akasaya 1	15	57	+	10	30	–	0	0	+ +
Raiden	11	31	+	13	42	–	0	0[b]	+ + +
Bonminori	17	48	+	15	67	–	3	4	+ +
Keburi	2	4	+ +	6	18	–	0	0	+ +
Enrei	15	53	+	12	23	–	1	3	+ + +
Tachisuzunari	9	28	+	4	5	–	0	0	+ +
Suzuyutaka	9	26	+	6	9	–	0	0	+ +
Miyagishirome	11	55	+ +	8	32	–	2	2	+ +
Aohata	7	13	+ +	4	7	–	0	0[b]	+ + +
Kurumimame	4	9	+ +	3	7	–	0	0	+ + +
Sakagami 2	12	35	+	3	5	–	0	0	+ + +
Nattou kotsubu	6	15	+	6	13	–	0	0	+
Usuao	6	8	– +	2	13	–	0	0	+ +
Tsukui-zairai 5	11	31	+	1	1	–	0	0	+ +
Asahi	15	58	+	11	32	–	0	0	+ +
Yamabe-daizu	18	60	+	5	11	–	0	0	+ +
Aonibu	14	24	+	7	10	–	0	0	+ +
Akishirome	17	101	+	7	17	–	0	0	+ +
Fukuyutaka	9	41	+	2	2	–	0	0	+ +
Total	279	1,082		234	783		7	11	

[a] Cs = soft callus, Cr = root genic, Cc = compact callus: – = no callus but only root formation, – + = little, + = medium, + + = large, + + + = very large

[b] Shoot primordia were differentiated

mones) for further development. Following enough development, especially in rooting, plantlets were transplanted to soil: peat moss: vermiculite = 1 : 1 : 1 mixture in a greenhouse following Barwale et al. (1986). Plants which grew to set seeds were scored as mature plants.

Results

In all of the varieties used, somatic embryos were formed on cultured immature embryos (Table 2). When induced by 2 mg/l 2,4-D, they formed exogenously around the surface of the immature embryos showing the globular, characteristic horn or amorphous shapes (Fig. 1 a). But when induced by 10 mg/l NAA, somatic embryos were formed both endogenously via callus-like brownish tissue (Fig. 1 b), or exogenously (Fig. 1 c), having more definitive cotyledons instead of the horn-like ones induced by 2,4-D. Long hypocotyls were present and characteristic of normal embryos.

The number of somatic embryos formed 6 weeks after culture initiation are shown in Table 2. On the medium containing 2 mg/l 2,4-D, the total number of somatic embryos from 20 immature embryos varied among the varieties continously from 133 of "*G. gracilis* T34", 101 of "Akishirome" and 72 of "Masshokutou (kou 503)" to 4 of "Keburi", at an average of 41.6. On the medium containing 10 mg/l NAA, the embryos varied from 179 of "Okuharadaizu", 81 of "*G. gracilis* T34" and 77 of "Masshokutou (kou 503) to 3 of "*G. gracilis* T135–590", 2 of "Fukuyutaka" and 1 of "Tsukuizairai 5" at an average of 30.1.

However, on the medium containing both 10 mg/l NAA and 0.1 mg/l BA, somatic embryos were barely formed in only 4 genotypes: 4 of "Bonminori", 3 of "Enrei", 2 of "*G. gracilis*" and 2 of "Miyagishirome". Callus formation, however, was generally vigorous.

"*G. gracilis*", "*G. gracilis* T34", "Masshokutou (kou 502)" and "Masshokutou (kou 503)" possessed

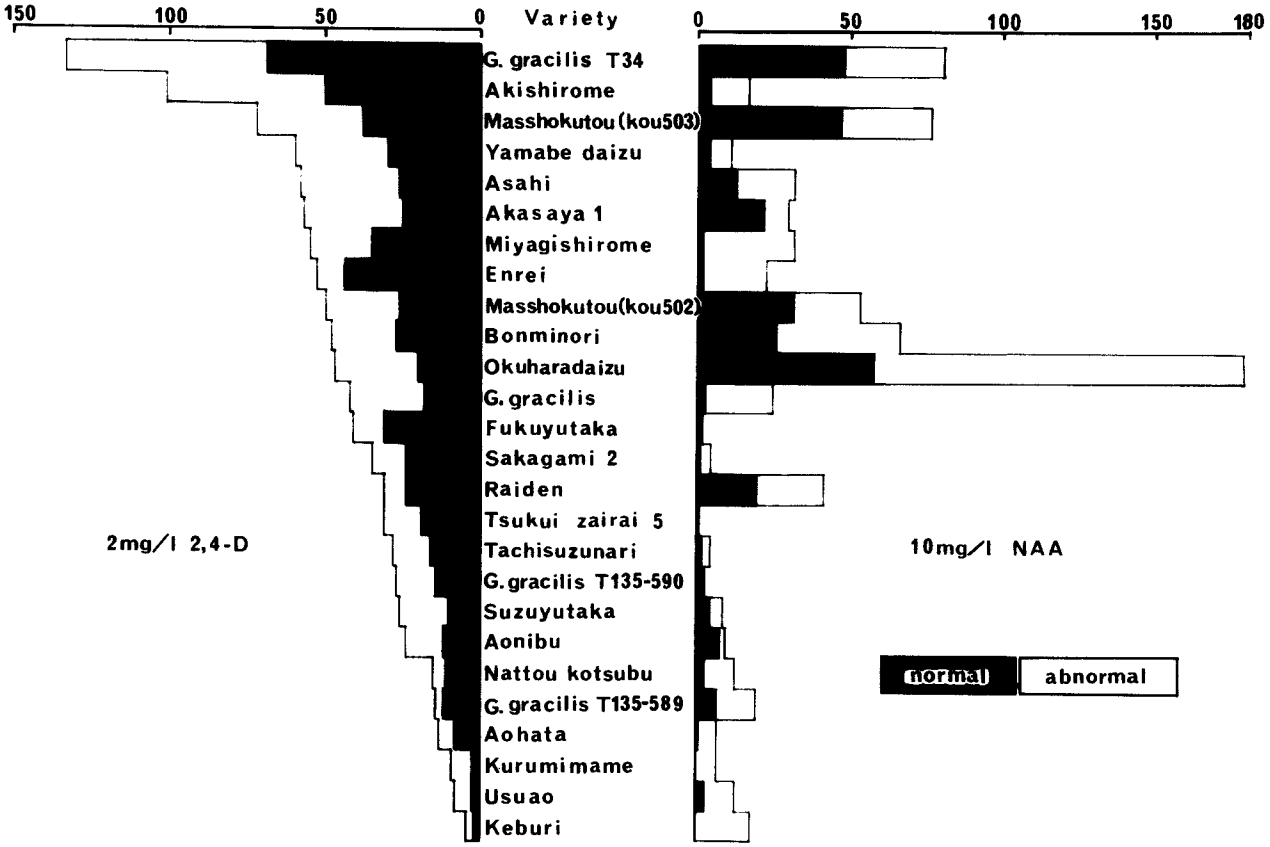

Fig. 2. Number of somatic embryos induced from each of 20 immature embryos cultured on media containing either 2 mg/l 2,4-D or 10 mg/l NAA

Table 3. Plant regeneration from somatic embryos within eight soybean varieties. Germination and plant maturation were estimated after 8 months of embryo induction. ( ) = percentage of embryos germinated/subcultured monthly

Genotype	Embryo selected	Subcultured monthly	Embryo germinated	Mature plant
G. gracilis	28	17	5 (29%)	3
G. gracilis T34	104	66	23 (35%)	11
Masshokutou (kou 502)	64	40	23 (58%)	6
Masshokutou (kou 503)	79	39	8 (21%)	0
Okuhara-daizu	81	54	5 (9%)	0
Bonminori	33	24	2 (8%)	1
Enrei	27	13	1 (8%)	0
Akishirome	126	109	0 (0%)	0

high competence of germination from somatic embryos at 29%, 35%, 58% and 21%, respectively (Table 3). On the other hand, germinations were limited in the commercial varieties such as "Okuhara-daizu", "Bonminori", "Enrei" and "Akishirome", although they possessed a higher competence of embryogenesis.

Discussion

Somatic embryos from 26 tested varieties were classified as normal types or abnormal types, according to their morphological shapes. In our study, we discriminated globular, heart-shaped, torpedo-shaped, and horn-shaped ones that possessed a hypocotyl and cotyledon(s). Lazzeri et al. (1985) showed that somatic embryos generated by NAA were more normal shaped than those generated by 2,4-D. By our calculations, however, the number of normal and abnormal types were almost equal in general varieties (Fig. 2). We calculated the normal plus abnormal types as the total somatic embryos based on the assumption that abnormalities of somatic embryos were due to a failure in normal expression, as we compared the embryogenic competence of 26 varieties using total somatic embryos. Several prominent genotypes were found as follows: (1) "*G. gracilis* T34", "Masshokutou (kou 502)" and "Masshokutou (kou 503)" frequently responded to both NAA and 2,4-D; (2) "Okuhara Daizu" frequently responded to NAA; (3) "Akishirome" frequently responded to 2,4-D; (4) "Keburi" and "Usuao" scarcely responded to either auxin.

Immature embryos of type (4) were more callusgenic than somatic embryogenic. Even if small somatic embryos were induced, they had a tendency to fail to become embryos, but to become callus (Fig. 1 d). No additional somatic embryos were generated from the white and soft calli which were derived from the primary tissue. Although those calli could generate many globular or heart-shaped embryoids after transference to suspension culture of MS medium lacking growth regulators, those embryoids failed to develop to torpedo stage or to germinate (data not shown). These phenomena suggest that the directions for callusgenesis and embryogenesis are different, and that embryogenesis from the completely dedifferentiated callus may be difficult.

The medium containing 0.1 mg/l BA and 10 mg/l NAA generated compact callus vigorously but strongly reduced embryogenesis (Table 2). The somatic embryos formed here were more normal in shape than those formed on 10 mg/l NAA alone. We previously reported the normal shaped, but very scarce, somatic embryogenesis in soybean under the presence of 0.01–0.1 mg/l BA with 1.0–10 mg/l NAA (Komatsuda and Ohyama 1986). In that study, in cv "Bonminori", only normal shaped somatic embryos consisting of a long hypocotyl and a pair of cotyledons were formed. But the percentage of cultures forming somatic embryos was very low (below 10%). The influence of BA on both frequency and morphology in soybean embryogenesis was reproducible in this study. The effect of cytokinin on somatic embryogenesis requires further investigation.

The germinating competence of somatic embryos was determined by the genotypes (Table 3). Somatic embryos with normal shape were transferred to subculture for germination (Fig. 3 a, b). The difference of auxins, NAA (10 mg/l) or 2,4-D (2 mg/l) in induction medium, did not contribute to the high frequency of germination in *"G. gracilis* T34" and "Masshokutou (kou 502)" (data not shown). In contrast to those two small-seed varieties, the germination frequencies of "Okuhara-daizu" and "Akishirome" were very low at 9% and 0%, respectively, where somatic embryos of "Okuhara-daizu" were induced mainly by NAA and somatic embryos of "Akishirome" were induced mainly by 2,4-D (Table 2). This indicates that genotypes contribute to germination, but germination is not affected by the kind of auxin used to induce embryogenesis.

However, after germination the embryos induced by NAA were superior to those induced by 2,4-D in further development of both shoots and roots. Somatic embryos induced by 2,4-D tended to fail in elongation of stem or roots. This clearly demonstrates the general aspects of auxin effects in soybean tissue culture, namely that NAA accelerates the development of both shoots and roots from tissue but 2,4-D inhibits them strongly. It could be said that shoot development to maturation is strongly affected by the kind of auxin supplemented in the medium for embryo induction.

For efficient development it was very important for somatic embryos to be transferred to three different media in correct sequence as described in "Materials and methods". If the first medium, which contained BA and IBA (Fig. 3 a), was omitted, somatic embryos were not developed. If the second medium (Fig. 3 b) was omitted, they were dedifferentiated, indicating that the second liquid medium not only accelerated the development of embryos but also absorbed extensive growth regulators from the first medium with somatic embryos. If the third medium was omitted, somatic embryos did not develop or germinate to shoot formation, indicating that growth regulators were necessary in culture medium for somatic embryos until they succeeded in recovering complete plantlets.

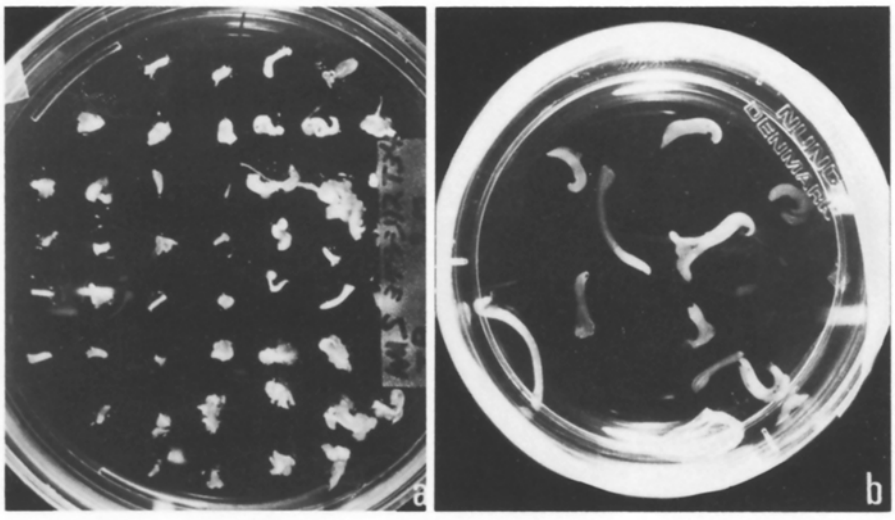

Fig. 3 a, b. Development of somatic embryos of soybean. **a** Buds and roots induction from somatic embryos of *"G. gracilis* T34" by 0.2 mg/l BA and 0.5 mg/l IBA on solid medium. **b** Liquid culture of somatic embryos

Regenerated plants cultured in the greenhouse grew to maturity. Some of the regenerated plants displayed twisted morphology and vigorous growth. Other plants showed variations mainly in leaf structure. Morphogenesis of soybean is well known to be strongly influenced by environmental conditions such as light intensity, photoperiod and temperature. As all of the regenerated plants were completely fertile to set pods and seeds, it could be proven that the variations were either genetical or physical.

The highly regenerative genotypes, *"G. gracilis* T34", *"G. gracilis"*, "Masshokutou (kou 502)" and "Masshokutou (kou 503)" (Table 3) could clearly be distinguished from Japanese commercial varieties by their semi-twisted forms and by black or brownish small seeds. Two of them, *"G. gracilis* T34" and *"G. gracilis"*, belonged to *Glycine gracilis,* a semi-wild relative of soybean in the taxonomic area between *Glycine soja* and *Glycine max.* However, *Glycine gracilis* now belongs to *Glycine max.* Kameya and Widholm (1981) showed that only *G. canescens* and *G. tomentella* were capable of plant regeneration from hypocotyl sections. They also showed, after testing eight Glycine species, that *G. canescens* was capable of plant regeneration from cotyledonary sections. Newell and Luu (1985) succeeded in plant regeneration from protoplast of *G. canescens.* These results coincide with our results and suggest that wild relatives of soybean, especially *G. canescens,* preserve high competence in plant regeneration.

The genotypes of high plant regeneration competence described in this report are useful for analysis of physiological and genetical factors controlling embryogenesis and organogenesis in soybean. Therefore, these varieties can also be applied to cell and protoplast culture, cell fusion and pollen culture for plant improvement.

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