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Somatic embryogenesis and plant regeneration from embryogenic callus of soybean, *Glycine max* L.

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ABSTRACT

During induction of somatic embryogenesis from immature embryos of soybean, a smooth-shiny and a rough callus were obtained. The smooth-shiny type developed from callus derived from cotyledonary tissue and cultured on media containing 10 mg/l 2,4-D. The rough type was derived from immature embryos, cotyledons, hypocotyls and hypocotyl segments from germinated seedlings on a medium containing various growth regulators. Plants were obtained from the smooth-shiny type but rough callus did not differentiate into plants. The conditions for formation of both callus types and regeneration of mature soybean plants from the smooth-shiny type were defined.

ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indoleacetic acid; IBA, indolebutyric acid; ABA, abscicic acid; GA3, gibberellic acid; BA, benzyladenine; B5, Gamborg et al., LS, Linsmaier & Skoog; MS, Murashige & Skoog; P, proline.

INTRODUCTION

Soybean plant or shoot regeneration methods from explants consisting of totipotent cells such as shoot tips (Kartha et al., 1981), cotyledonary nodes (Cheng et al., 1980) and stem nodes (Saka et al., 1980) have been achieved. Development of embryo-like structures in suspension culture from hypocotyl and ovaries of soybean was shown by Beversdorf and Bingham (1977). Somatic embryos from cell suspension culture were observed by Phillips and Collins (1981). Kameya and Widholm (1981), Widholm and Rick (1983) and Grant (1984) reported plant regeneration from cultures of the wild perennial species Glycine canescens.

Somatic embryogenesis has been observed in cell suspension culture of soybean (Christianson et al., 1983). High frequency of somatic embryogenesis has been reported by Lippmann and Lippmann (1984). Ghazi and Nabors (1986) described a similar system and have studied embryo development associated with types of embryogenic callus. Consistent regeneration of soybean plants from callus was recently reported (Ranch et al., 1985).

In this paper two morphological types of embryogenic callus, the origin and duration of their differentiation and subsequent plant regeneration from soybean cultures are described.

MATERIALS AND METHODS

Plant materials

At Colorado State University seedlings were obtained from seeds of Glycine max (L.), cv. Prize. At Punjab University, mature and juvenile leaf segments and stem segments were obtained from plants of cv. FFR, seeds of which were provided by I. K. Smith, Department of Botany Ohio University.

Experimental

At Punjab University seeds of FFR 335 were surface sterilized in 0.25% solution of mercuric chloride for 5 to 8 minutes after which they were rinsed repeatedly and planted on Knop's (1869) medium. Mature and juvenile leaf segments and stem segments with or without nodes were excised and cultured on MS media containing 2% sucrose and 0 to 7 mg/l 2,4-D with or without 1 mg/l kinetin.

At Colorado State University seeds of cv. 'Prize' were surface sterilized in 75% ethanol followed by immersion in 8% commercial bleach containing 5.25% sodium hypochlorite solution plus two drops of Tween 20. The solution was stirred under vacuum for 15 minutes. The solution for sterilization was removed by 3 to 4 rinses with sterile distilled water. Germination was accomplished on a semi-solid basal medium solidified with 0.8% agar and containing LS major and minor salts, vitamins and 2% sucrose. The hypocotyls of the seedlings 1,3 and 5 cm long, were removed from 1 to 5 mm below the cotyledonary attachments and sectioned to lengths of 3 mm.

Whole immature embryos were obtained from pods 2 to 10 cm long produced by plants grown in the greenhouse. Embryos were 2 to 10 mm long. Cotyledons and hypocotyls were excised as segments of the embryo. The meristematic sections of the segments were discarded; the hypocotyl and cotyledons were retained. The whole embryos and the embryo segments were sterilized in a manner similar to the mature seeds, however, with only 10 minutes of stirring.

Hypocotyls (from seedlings), embryos and embryo segments were planted on a solid basal medium (0.7% agar) containing LS major and minor salts, plus

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vitamins and 2% sucrose and various concentrations of growth regulators. The pH was adjusted to 5.8 prior to autoclaving. Cultures were kept under continuous light at 28±2°C with illumination provided by four General Electric wide spectrum florescent lamps providing an intensity of approximately 3500 Lux.

Variables investigated included hormone type and concentration, nitrogen source, proline requirement and developmental stage of the explant. Various concentrations of IAA in combination with BA as well as 2,4-D in combination with BA were tested for potential as culture initiation media (Table 1). Also 2,4-D and proline combinations, nitrogen source (NH_4NO_3 and NH_4Cl), GA_3, zeatin, IBA and ABA were tested as components of maintenance and regeneration media (Table 1).

At the end of four weeks, the callus obtained from different sizes of cotyledonary segments cultured on media containing 10 mg/l 2,4-D or 10 mg/l 2,4-D in combination with 0.132 to 0.264 mg/l ABA, were transferred to the same respective fresh medium. In addition, pieces of calli (15 mg), were transferred to a series of media for maintenance studies (Table 1) in which the effect of nitrogen source and proline on callus maintenance was tested. The growth regulator selected for maintenance media in this latter series was 2,4-D because our previous observations indicated its effectiveness in producing embryogenic callus.

Ten cultures (10 ml vials, one explant per vial) were scored for each set (21 sets) of conditions. Every four weeks visual observations were made, and transfer to fresh media was performed except for cotyledonary segments which required observation more frequently. All experiments were repeated three times.

RESULTS AND DISCUSSION

It was observed that callus from all explant sources produced on media containing 2,4-D was creamish in color, and friable. Callus iniatiated from whole embryos, cotyledons, hypocotyl segments and hypocotyls from germinated seedlings on media containing IAA was greenish and more compact. Also these four explant sources on media containing NH_4Cl as a nitrogen source appeared to produce more vigorously growing callus, compared with media containing NH_4NO_3. Proline had no apparent effect on callus growth.

Two types of embryogenic callus were observed. One type appeared smooth-shiny (Fig. 1a) and was produced from cotyledonary segments on media containing 10 mg/l 2,4-D or 10 mg/l 2,4-D in combination with 0.132 and 0.265 mg/l ABA. The other type of embryogenic callus was consistently rough as shown in Fig. 1b, and was produced from whole embryos, cotyledons, hypocotyls, and hypocotyls from germinated seedlings on all initiation media except 10 mg/l 2,4-D. There was an inverse relationship between length of hypocotyl segments obtained from seedling and induction frequency of rough embryogenic callus. The smooth-shiny embryogenic callus was greenish and translucent while the rough embryogenic callus was opaque and creamish in color. The greatest amount of smooth-shiny embryogenic callus was produced from cotyledons 6 mm long (Table 2). When 2,4-D was used in combination with 0.264 mg/l ABA, normal development of smooth-shiny embryogenic callus occurred.

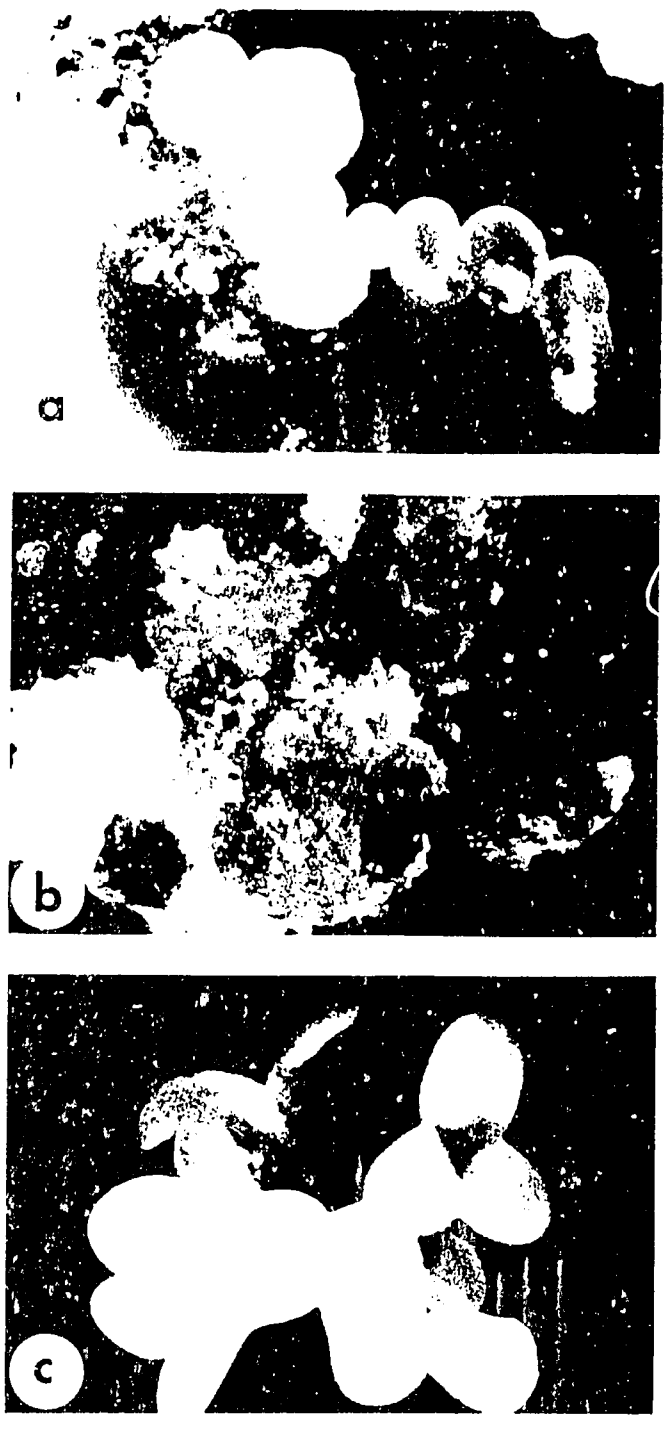

Fig. 1 Regeneration of soybean plants Glycine max c.v. Prize

(a) Smooth-shiny embryogenic callus from cotyledonary segments, formed on media containing 10 mg/l 2,4-D.
(b) Rough embryogenic callus appeared in cultures as compacted callus.
(c-e) Different initiation patterns of embryos from smooth-shiny embryogenic callus.
(f) Shoot differentiated simultaneously with root formation.
(g) Regenerated plants.

d

e

f

g

After two to three weeks incubation in the dark in regeneration medium (Table 1), three different patterns of developing embryos from smooth-shiny embryogenic callus (Fig. 1c-e), and also a few shoots with roots (Fig. 1f) were produced. Complete plantlets were obtained from smooth-shiny embryogenic callus when cultured on media containing 0.104 mg/l GA_3 or 0.110 mg/l zeatin (Table 3). However, some of the smooth-shiny embryogenic callus produced only roots and some only shoots when cultured on the above media. Shoots or roots alone, from smooth-shiny embryogenic callus were also obtained from cultures on media containing ABA plus either GA_3 or zeatin (Table 3). When these shoots were subcultured on B5 medium supplemented with 0.122 mg/l IBA (Tilton and Russell, 1984), or LS media combined with 5 mg/l IBA, roots were obtained.

Table 1. Hormonal composition of LS media for embryo initiation, maintenance and plant regeneration from various explants of soybean cv. Prize.

Initiation media (mg/l)			
1.	1 IAA	11.	1 2,4-D + 0.5 BA
2.	1 IAA + 0.5 BA	12.	1 2,4-D + 5 BA
3.	1 IAA + 5 BA	13.	2 2,4-D
4.	2 IAA	14.	2 2,4-D + 0.5 BA
5.	2 IAA + 0.5 BA	15.	2 2,4-D + 5 BA
6.	2 IAA + 5 BA	16.	5 2,4-D
7.	5 IAA	17.	5 2,4-D + 0.5 BA
8.	5 IAA + 0.5 BA	18.	5 2,4-D + 5 BA
9.	5 IAA + 5 BA	19.	10 2,4-D
10.	1 2,4-D	20.	10 2,4-D + 0.132 ABA
		21.	10 2,4-D + 0.265 ABA

Maintenance media (mg/l)			
22.		+ 0.2 2,4-D + 0	Proline
23.		+ 0.2 2,4-D + 25 mM	Proline
24.		+ 0.5 2,4-D + 0	Proline
25.		+ 0.5 2,4-D + 25 mM	Proline
26.	minus	+ 0.2 2,4-D + 0	Proline
27.	NH_4NO_3	+ 0.2 2,4-D + 25 mM	Proline
28.		+ 0.5 2,4-D + 0	Proline
29.		+ 0.5 2,4-D + 25 mM	Proline
30.	minus NH_4NO_3	+ 0.2 2,4-D +0	Proline
31.	plus 800	+ 0.2 2,4-D + 25 mM	Proline
32.	NH_4Cl	+ 0.5 2,4-D + 25 mM	Proline
33.		+ 0.5 2,4-D + 25 mM	Proline

Regeneration media (mg/l)	
34.	No growth regulators (control)
35.	0.104 GA_3
36.	0.103 GA_3 + 0.132 ABA
37.	0.103 GA_3 + 0.132 ABA + 0.102 IBA
38.	0.110 zeatin
39.	0.110 zeatin + 0.132 ABA
40.	0.110 zeatin + 0.132 ABA + 0.102 IBA
41.	0.110 zeatin + 0.132 ABA + 0.103 GA3

Rooted plantlets were transferred to three inch pots containing vermiculite and grown in a growth chamber. Subsequently, after establishment, plants were transferred to larger pots of soil in the greenhouse. To date, 21 plants were grown to maturity in the greenhouse (Fig. 1g).

Low concentrations of 2,4-D produced rough embryogenic callus however, high concentration of 2,4-D produced smooth-shiny embryogenic callus in a relatively shorter time (Fig. 2).

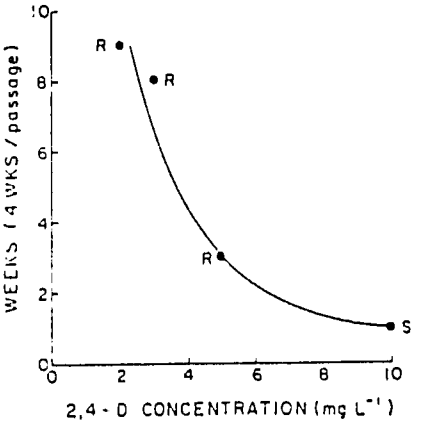

Fig. 2. Relationship between time interval for embryogenic callus formation and 2,4-D concentration. Each coordinate represents at least 30 cultures. "S" represents smooth-shiny and "R" represents rough.

Table 2. Formation of smooth-shiny embryogenic callus from developmentally different cotyledonary segments on media containing 10 mg/l 2,4-D

Immature embryos length (mm)	Cultures with embryos (%)	Embryos per culture (No.)
2	-	-
3	6	0.06
4	36	2.04
5	59	2.00
6	39	2.42
7	38	1.23
8	44	2.00
9	25	1.00
10	13	0.13

Table 3. Percentage of cultures containing shoots, roots, and plantlets initiated from smooth-shiny embryogenic callus from cultured cotyledonary segments within 2 to 3 weeks. Each treatment involved 30 somatic embryos.

Growth regulators[1]	Shoots (%)	Roots (%)	Plantlets (%)
Zeatin+ABA+GA3	50	10	-
Zeatin+ABA	60	20	10
Zeatin	60	20	20
Zeatin+IBA+ABA	65	-	-
GA3+ABA+IBA	70	-	-
GA3+ABA	55	20	-
GA3	55	25	20
Control	30	-	-

[1] See Table 1, regeneration media

When immature leaves of FFR 335 were cultured on 2,4-D enriched media, the embryogenic callus changed to the globular stage and remained without further development (Fig. 3a). After four months with 0.1 mg/l 2,4-D plus 1 mg/l kinetin, shoots

differentiated in 4% of the cultures (Fig. 3b) but further development did not occur. Stem segments, including nodes, cultured with 4 to 5 mg/l 2,4-D were observed to swell along their lengths, form callus from both ends and produce small possibly proembryonic structures (Fig. 3c).

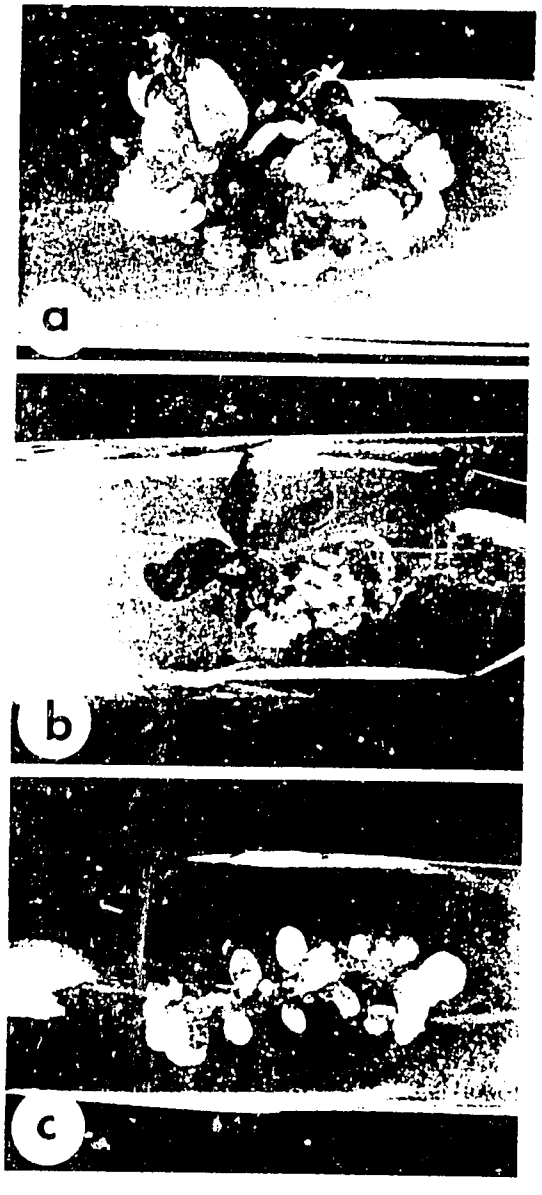

Fig. 3. Embryogenesis and plantlet of soybean, Glycine max cv. FFR 335 from callus. (a) Leaf callus (b) Regenerated plantlet from leaf callus (c) stem callus

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