

PLANT REGENERATION FROM EMBRYOGENIC SUSPENSION CULTURES OF CHINESE YAM (*DIOSCOREA OPPOSITA* THUNB.)

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A competent, embryogenic suspension culture of Chinese yam (*Dioscorea opposita* Thunb. cv. 'Nagaimo') has been obtained. Embryogenic callus was induced from stem segments cultured on an agar-solidified MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). One month following placement of the embryogenic callus in a liquid medium containing 2,4-D, the embryogenic tissue began to proliferate rapidly. Established suspension cultures consisted almost entirely of early-stage pro-embryos with very little contamination from non-embryogenic tissues. Under optimum conditions, suspension culture packed cell volume increased 2.5-fold per week. Following transfer of the tissue to a hormone-free medium, the embryogenic tissue developed. Globular embryos were formed within 4 weeks and addition of benzyl adenine further enhanced development and germination. Plantlets were regenerated by culturing embryos on a hormone-free agar-solidified medium.

Key words: chinese yam; *Dioscorea opposita*; suspension culture; embryogenesis; regeneration

Introduction

Chinese yam (*Dioscorea opposita* Thunb.) is a monocotyledonous, dioecious crop grown in far east countries for human consumption. The tubers of some Chinese yam varieties grow vertically in sandy soil under temperate conditions and can reach a length of 1 m.

The use of cross-breeding techniques has been limited for crop improvement of Chinese yam. Zygotic embryos, formed by sexual hybridization, do not develop or germinate in vivo [1]. Because of this barrier to

sexual propagation, Chinese yam is strictly vegetatively propagated. This has resulted in transmission of virus diseases and a low propagation rate. To alleviate these problems, tissue culture techniques, such as stem tip culture, micropropagation [2], and in vitro bulbil formation [3] have been developed.

Although somatic embryogenesis in Chinese yam has never been described, two other *Dioscorea* species [4,5] have been reported to form somatic embryos. Somatic embryoids of *D. deltoidea* [4] were induced from tuber-derived callus cultured on media containing 2,4-D and kinetin. Somatic embryos were obtained, but proliferation of embryogenic tissue and plant regeneration were not described. In the second report on somatic embryogenesis in one of the sexually-fertile *Dioscorea* species, *D. floribunda* [5] somatic embryos were obtained from seed-derived callus cultured on media containing only 2,4-D. Although embryogenic tissue pro-

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid; BA, 6-benzyladenine.

liferated in suspension culture and plants were regenerated, growth of the embryogenic suspension culture rapidly declined after several subcultures and the suspensions could not be maintained.

Our research provides the first report of somatic embryogenesis in *D. opposita*. This is also the first report of a maintainable, regenerable embryogenic suspension culture of any *Dioscorea* species. The procedures for induction of embryogenic callus, proliferation of embryogenic tissue in liquid suspension culture, and development and germination of somatic embryos are described.

Materials and methods

The basal medium used for all experiments contained MS salts [6], B5 vitamins [7], and 3% sucrose. Plant growth regulators (2,4-D, NAA, or BA) were added to the medium before adjusting the pH to 5.8 and, in the case of solid media, agar (0.8%) was added before autoclaving. All cultures were maintained at 28°C. Cultures which were maintained under light conditions were illuminated with a 16 : 8-h light/dark photoperiod with a light intensity of 30 $\mu\text{E}/\text{m}^2\text{s}$.

Maintenance of the virus-free clone

Nodal stem segments from a stem tip-derived, virus-free clone of Chinese yam (*Dioscorea opposita* Thunb. cv. 'Nagaimo') established as in [3] were excised and cultured for axillary bud proliferation. Each segment (containing one axillary bud) was cut to 10 mm and placed in a 25 × 150 mm test tube containing 10 ml of the liquid basal medium supplemented with 0.02 mg/l BA. After 2–3 months of stationary culture, shoots from the axillary buds were again divided into nodal stem segments and subcultured. This virus-free clone has been maintained for more than 2 years, with a multiplication rate of 5–7-fold during every 2–3-month subculture period.

Induction of embryogenic callus

Stem segments from the virus-free clone

were excised for induction of embryogenic callus. Segments without axillary buds were cut into 10-mm lengths and placed on agar-solidified induction media containing either 0, 0.3, 1.0, 3.0, or 10 mg/l 2,4-D. For each treatment, 54 total stem segments were cultured in six petri dishes (25 × 100 mm), each containing 40–50 ml of medium. Three culture dishes were placed under light conditions, and the remaining three dishes were placed in the dark.

All cultures were transferred to fresh induction medium every 2 months and embryogenic callus formation was evaluated 6 months after culture initiation. Embryogenic calli were then either used for establishment of suspension cultures, or maintained on the agar medium with bimonthly transfers.

Initiation and subculture of embryogenic suspension cultures

Three to five pieces of embryogenic callus (each approximately 1 mm³) were placed in a 125 ml deLong flask containing 35 ml of liquid medium supplemented with 0, 0.3, 1.0, or 3.0 mg/l 2,4-D. For each medium, six flasks (three in the light and three in the dark) were prepared. All liquid cultures were agitated at 150 rev./min. After 2 months of liquid culture, embryogenic cell clumps (0.1–4 mm³) were isolated under a dissecting microscope and subcultured. A single 1-mm³ clump could be transferred to 35 ml of fresh medium to establish a new culture.

After suspension cultures were established, the embryogenic tissues were subcultured without rigorous visual selection. For subculture, 0.1 ml packed cell volume (PCV) of embryogenic tissue was transferred to 35 ml of fresh medium. To measure this low PCV, the suspension culture tissue was taken up with a 1-ml wide mouth pipet. The tip of the pipet was then placed squarely on the bottom of the flask and the medium was blown out until air bubbles escaped. PCV was recorded by reading the calibrations on the pipet. To resuspend the tissue, fresh liquid medium was taken up into the pipet.

One week following transfer to fresh medium, total PCV was recorded and 0.1 ml PCV was again subcultured into 35 ml of fresh medium.

To optimize growth of the embryogenic suspension culture tissue, 0.1 ml PCV of isolated embryogenic tissues grown in liquid medium (1.0 mg/l 2,4-D medium, dark) was transferred to liquid media containing five levels of 2,4-D (0, 0.1, 0.3, 1.0 and 3 mg/l) both in the light and in continuous darkness. Following a 2-week "conditioning" period, the PCV of three flasks was measured weekly for 6 weeks.

Development and germination of somatic embryos

Embryogenic tissues from four treatments (0.3 and 1.0 mg/l 2,4-D; light and dark) were transferred into a hormone-free liquid medium to evaluate the potential for embryo development. A 0.1-ml PCV inoculum of proliferating embryogenic tissues from each treatment was transferred to the hormone-free liquid medium and cultured in the light. The liquid medium was replaced with fresh hormone-free medium weekly for 4 weeks. Following this time, the mature somatic embryos in each flask were counted.

In another experiment, embryogenic tissues from 0.3 mg/l 2,4-D medium grown under light conditions were subcultured into the hormone-free liquid medium for 4 weeks with weekly replenishing of medium. Developing (but still globular) somatic embryos were then subcultured to liquid media containing six different levels of BA (0, 0.1, 0.3, 1.0, 3.0 and 10 mg/l). After 4 weeks of culture under light conditions, the number of germinating embryos in each flask was recorded.

For embryo development on an agar-solidified medium, embryogenic tissues from the proliferation medium (0.3 mg/l 2,4-D; light) were transferred onto media containing various combinations of NAA and BA (both at 0, 0.3, 1.0 and 3.0 mg/l). For each treatment, 81 embryogenic cell clumps (9×9 grid), each 1

—2 mm in diameter, were cultured in a plastic petri dish with 40–50 ml of agar-solidified medium. After 2 months, mature embryos were transferred to the hormone-free agar medium for embryo germination.

Germinating embryos with well developed leaves and roots were transferred to a 2:2:1 (by vol.) mixture of vermiculite, peat moss and soil. After 2 weeks of acclimation, plantlets were transferred to the greenhouse.

Results

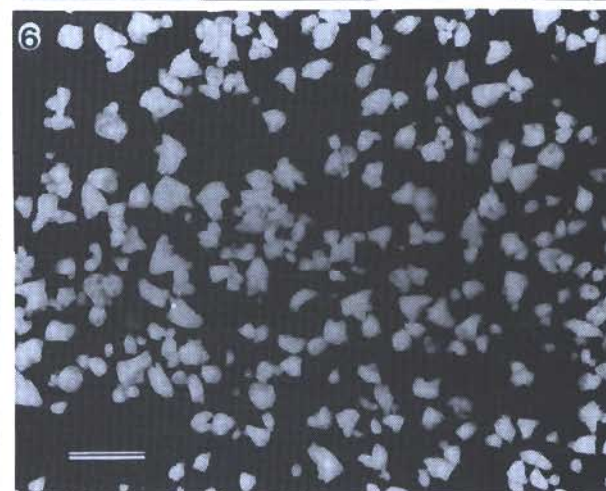
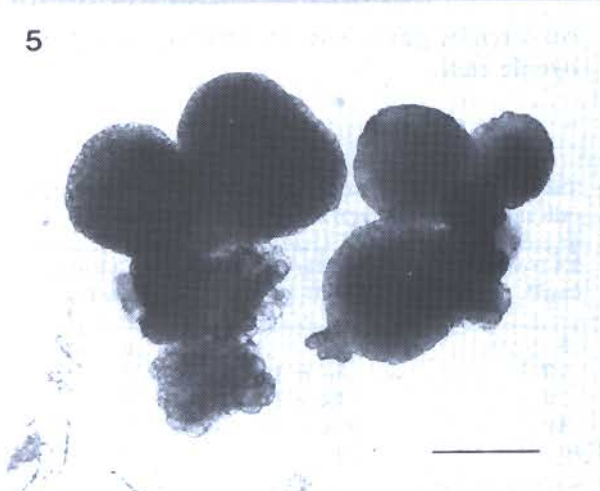
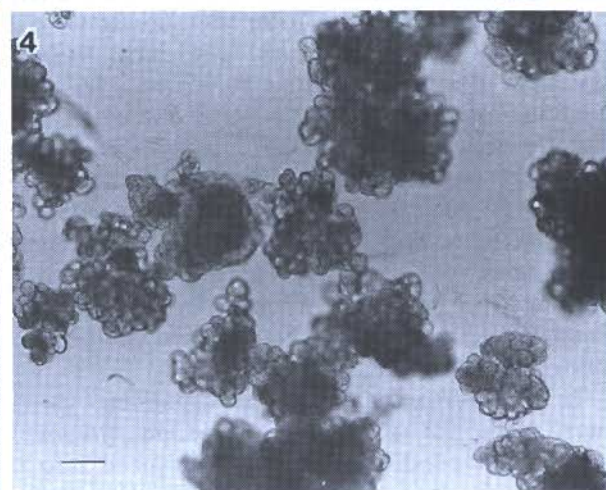
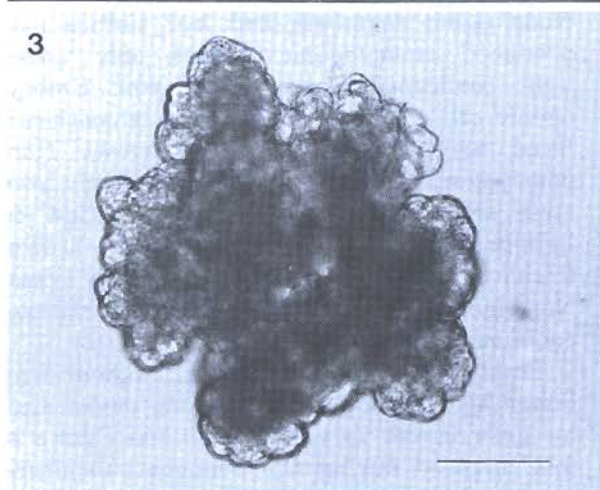
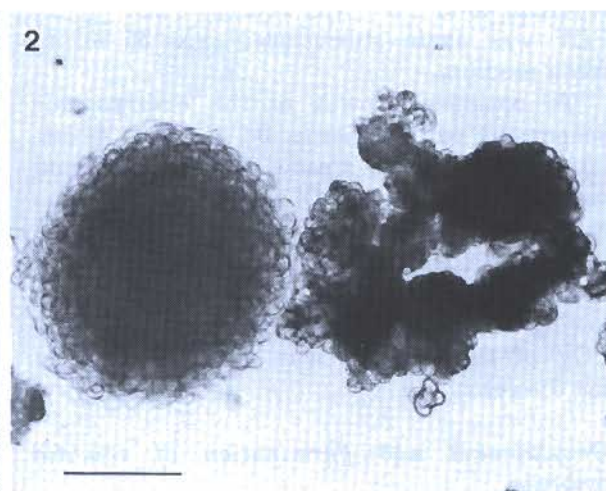
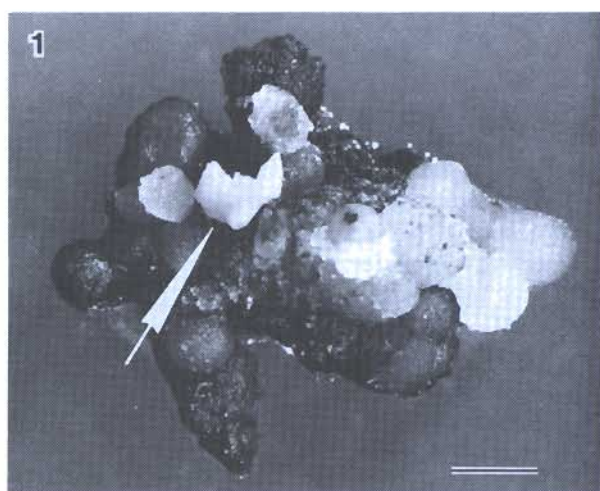
Induction of embryogenic callus

Embryogenic callus was obtained from stem segments after at least 3 months of culture and only under light conditions (Table I). Nodal stem segments and leaf tissues also produced embryogenic tissues only under light conditions (data not shown). Embryogenic calli were typically white, smooth-surfaced and compact (Fig. 1, arrow). Non-embryogenic calli were also induced from stem segments and were either yellow to light-brown, nodular and compact; brown, friable and soft; or a mixture of both types. Non-embryogenic calli produced adventitious roots on the induction medium.

Embryogenic calli were subcultured bimonthly on the 2,4-D containing media. Callus growth was very slow with less than a 2-fold increase during the 2-month subculture period. In addition, the embryogenic calli continually gave rise to prolific, non-embryogenic calli.

Table I. Percent of stem segments forming embryogenic calli (mean \pm S.E.) after 6 months of culture.

2,4-D conc. (mg/l)	Cultured in light	Cultured in dark
0	0	0
0.3	3.7 \pm 3.7	0
1.0	7.4 \pm 3.7	0
3.0	37.0 \pm 7.4	0
10	0	0



Initiation and subculture of embryogenic suspension cultures

Within the first month after inoculation of embryogenic callus pieces into liquid media, both non-embryogenic (Fig. 2, left) and embryogenic (Fig. 2, right) tissues proliferated slowly. Non-embryogenic tissues were round, rough-surfaced, 2–3 mm in diameter and consisted of large, vacuolated cells (Fig. 2, left). Embryogenic tissues were irregularly shaped and consisted of clumps of small, highly cytoplasmic cells (Fig. 3). Following a 1-month lag period, the embryogenic tissues started to proliferate more rapidly. Embryogenic suspension cultures were successfully initiated and subcultured from the following five treatments: 1.0 and 3.0 mg/l 2,4-D both in the light and the dark and 0.3 mg/l 2,4-D in the dark.

If attempts were made to use 2,4-D concentrations of 0.1 mg/l or lower for proliferation of the embryogenic suspension culture, embryo development resulted. Use of 3.0 mg/l 2,4-D for suspension culture maintenance promoted non-embryogenic growth and reduced embryogenic growth. The 2,4-D concentrations which gave the best embryogenic growth were 0.3 and 1.0 mg/l. At these 2,4-D levels, the isolated embryogenic tissues proliferated uniformly and there was very little contamination from non-embryogenic cells (Fig. 4). Among the tested culture conditions for maintenance of the embryogenic suspension, 0.3 mg/l 2,4-D in the light gave

Table II. Relative growth rates of embryogenic suspension culture tissue under four different conditions and somatic embryo development from these suspension cultures.

2,4-D conc. (mg/l)	Light	Growth rate ^a (Mean ± S.E.)	Embryo development ^b (Mean ± S.E.)
0.3	Light	2.49 ± 0.06	12471 ± 1792
0.3	Dark	2.22 ± 0.10	16541 ± 784
1.0	Light	2.16 ± 0.05	14534 ± 1053
1.0	Dark	1.77 ± 0.09	22452 ± 588

^aRelative packed cell volume increase of the embryogenic tissues per week.

^bNumber of the somatic embryos (from 0.1 ml PCV embryogenic suspension culture tissue) developed after 4 weeks of culture in hormone-free liquid medium.

the highest proliferation rate, resulting in a 2.5-fold increase in PCV per week (Table II).

Development and germination of somatic embryos in liquid culture

Development of somatic embryos became obvious within 2 weeks after proliferating embryogenic tissues were transferred into the hormone-free liquid medium. The surface of the tissue became smooth and large dense clumps of developing embryos were formed. Within 4 weeks, globular and later-staged embryos had developed (Fig. 5). By 2 months, mature embryos (Fig. 6) were formed.

The number of embryos produced from proliferative embryogenic tissues was influ-

Fig. 1. Embryogenic callus (at arrow) formation from stem segment of Chinese yam cultured on agar-solidified medium containing 3.0 mg/l 2,4-D (Bar = 2 mm).

Fig. 2. Embryogenic suspension culture in medium containing 1.0 mg/l 2,4-D 1 month after establishment. Remnants of non-embryogenic tissue (left) are distinguished from proliferating embryogenic tissue (right). (Bar = 0.2 mm).

Fig. 3. High-magnification picture of embryogenic tissue of Chinese yam in suspension culture (Bar = 0.1 mm).

Fig. 4. Embryogenic tissue in suspension culture medium containing 0.3 mg/l 2,4-D. Note absence of non-embryogenic tissues (Bar = 0.2 mm).

Fig. 5. Formation of globular embryos 4 weeks following transfer to hormone-free liquid medium (Bar = 0.2 mm).

Fig. 6. Synchronous embryo development 8 weeks following transfer to the hormone-free liquid medium (Bar = 10 mm).

enced by the proliferation culture conditions (Table II). A 0.1 ml PCV of embryogenic tissues from the suspension containing 1.0 mg/l 2,4-D in the dark gave rise to more than 20 000 mature embryos following transfer to the hormone-free suspension medium.

Developing embryos underwent precocious germination without a maturation period.

Germination was enhanced by the addition of BA to the liquid suspension medium (Table III). Within 2 weeks of culture in BA containing medium, somatic embryos showed both root and hypocotyl elongation (Fig. 7). High BA concentrations (≥ 5 mg/l) promoted callus formation from the surface of somatic embryos. The germination rate was maxim-

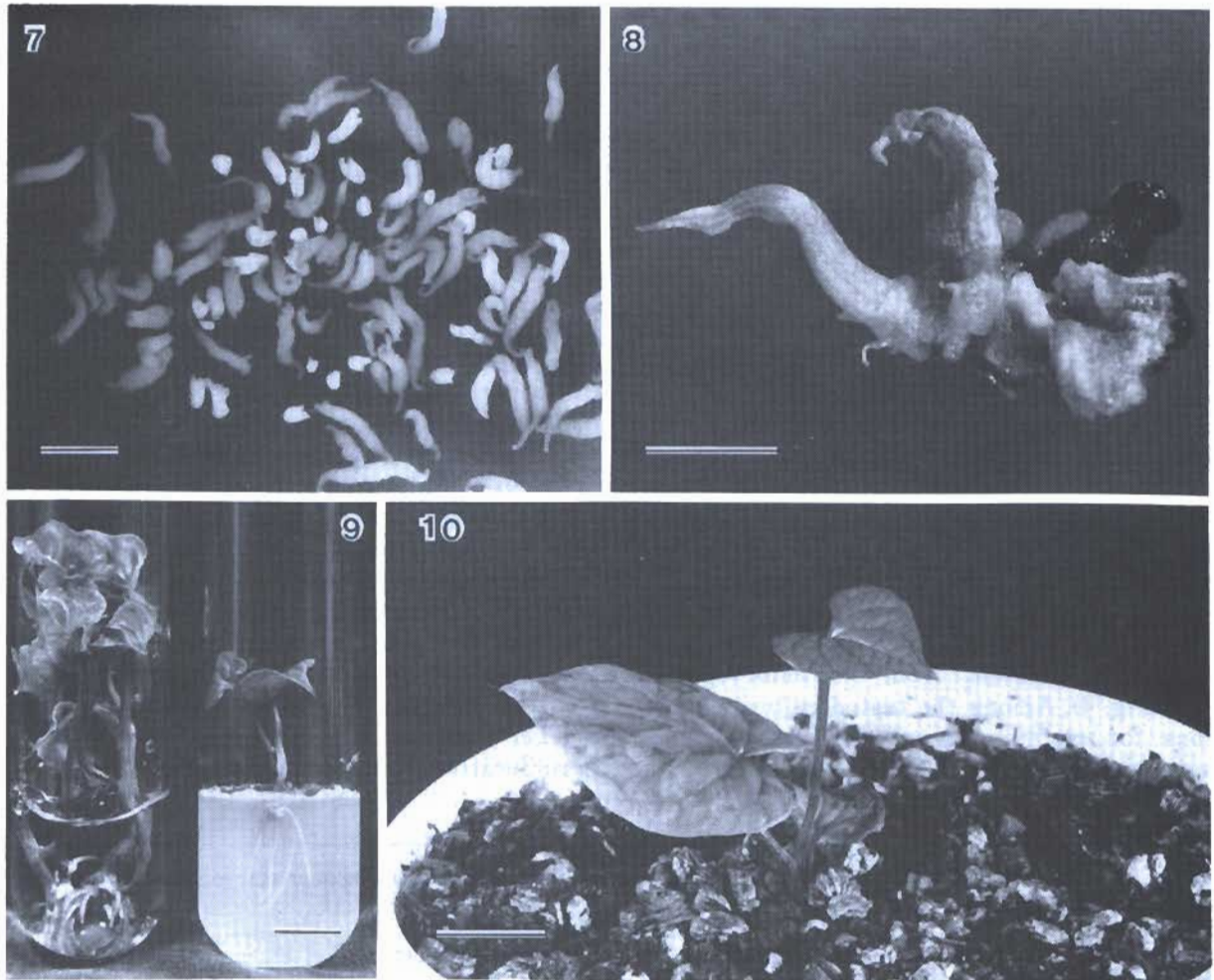


Fig. 7. Germinating embryos in liquid medium containing 0.3 mg/l BA (Bar = 4 mm).

Fig. 8. Germination of Chinese yam somatic embryo on an agar-solidified medium containing 3.0 mg/l BA (Bar = 4 mm).

Fig. 9. Somatic embryo-derived plantlets cultured on hormone-free agar medium (right) and liquid medium (left) (Bar = 10 mm).

Fig. 10. Regenerated plant of Chinese yam (Bar = 10 mm).

Table III. Effect of BA addition on embryo germination in liquid culture.

BA conc. (mg/l)	Germination* (%)
0	0.0 ± 0.0
0.1	1.9 ± 0.5
0.3	25.4 ± 3.5
1.0	43.1 ± 3.5
3.0	51.1 ± 10.7
5.0	30.2 ± 11.3
7.0	16.9 ± 4.8
10	17.7 ± 12.3

*Percent of embryos germinating after 4 weeks of culture (mean ± S.E.).

ized when 3.0 mg/l BA was used. Although embryo morphology was slightly abnormal, approximately 50% of the total embryos germinated using this treatment (germination was defined as root and hypocotyl elongation). Lower concentrations (0.3 or 1.0 mg/l) appeared to be more suitable for morphologically normal germination. Embryo germination in a liquid medium occurred at a very high frequency. However, regeneration of whole plants from embryos which developed and germinated in liquid culture was not obtained.

Plant regeneration

Embryogenic tissue, proliferating in the liquid medium containing 0.3 mg/l 2,4-D

Table IV. Number of plantlets obtained from embryogenic tissue cultured on NAA-BA containing agar-solidified media.

NAA conc. (mg/l)	No. of plantlets regenerated*			
	BA concentration (mg/l)			
	(0)	(0.3)	(1.0)	(3.0)
0	0	0	0	3
0.3	2	9	14	4
1.0	0	2	4	6
3.0	0	0	0	2

*Number of plantlets regenerated from 81 pieces of embryogenic tissue.

under light conditions, was transferred to the NAA-BA containing agar media for plant regeneration. Embryo development on agar media could not be observed and followed as easily as in the liquid suspension system. Elongation of the embryo axis was apparent 1 month after culture on NAA-BA containing agar medium. Most of the germinating embryos had turgid shoots, showed limited root elongation, but failed to develop further (Fig. 8) on the NAA-BA containing media.

Plant regeneration was obtained following transfer of some of these germinating embryos to a hormone-free, agar-solidified medium (Fig. 9, right). The number of plants obtained from embryos, which developed on the NAA-BA media, are shown in Table IV. Plant regeneration was highest overall when the embryos developed and germinated on a medium containing NAA and BA at 0.3 mg/l and 1 mg/l respectively. Competent embryos were also obtained on all media containing the highest BA concentration (3 mg/l BA) regardless of the NAA level. Regenerated plants could be subsequently multiplied by stem node propagation in liquid medium (Fig. 9, left). Plantlets with fully developed leaves and roots were transferred to the soil mixture and gradually exposed to ambient humidity (Fig. 10).

Discussion

Embryogenic callus cultures of Chinese yam were similar in morphology to other embryogenic monocot tissues [8]. Once embryogenic tissue was obtained, it could be easily selected and separated from non-embryogenic tissues. The difficulty with initiation and maintenance of the embryogenic callus was the length of time required for induction and subculture. Embryogenic callus was obtained only after 3 months of culture and following subculture, the doubling time of the tissue was over 2 months.

In contrast to the solid-support system, the tissue in suspension culture was very prolific. The doubling time was reduced to

less than 1 week and the tissue proliferated at a very early ontogenetic stage. A very low subculture inoculum (one 1 mm³ clump of embryogenic tissue into 35 ml of fresh medium) could be used to maintain embryogenic suspension cultures of Chinese yam. This low subculture density appeared to suppress growth of non-embryogenic tissues while permitting prolific growth of embryogenic tissues. Use of low inoculum subculture for maintenance of embryogenic suspension cultures has been reported for cotton [9] and soybean [10].

Embryogenic suspension culture tissue of Chinese yam grew rapidly in media containing 0.3 or 1.0 mg/l 2,4-D both under light and dark conditions. Although proliferation was highest in 0.3 mg/l 2,4-D under light conditions, the number of embryos which developed from this treatment was the lowest (Table II). Embryogenic tissues cultured in the light may be proliferating at a slightly later developmental stage than under dark conditions. Thus, for a given volume of tissue, there would be more early-staged (smaller) embryos than later-staged (larger) embryos. In addition, embryogenic tissues from the higher 2,4-D-containing medium may continue to proliferate in the hormone-free development medium due to a possible carry-over of 2,4-D.

The only other descriptive report on embryogenesis in any yam species is for *D. floribunda* [5]. *D. deltoidea* has been reported to form somatic embryoids and shoots from callus cultures but a distinction between shoot morphogenesis and embryogenesis in these cultures was never made. The procedures developed for induction and maintenance of embryogenic tissue of *D. opposita* (this study) and *D. floribunda* [5] are markedly different. Stem segments of Chinese yam were used as the explant source in this study while excised zygotic embryos or seedlings were used for induction of embryogenic callus in *D. floribunda* [5]. Availability of seeds or zygotic embryos from a sexual cross can sometimes be limiting [1], but stem seg-

ments can be easily maintained using a stem node propagation system [11,12]. Another clear difference between the two systems was the induction response under light and dark conditions. Chinese yam formed embryogenic calli only under light conditions while dark incubation was used for induction of embryogenic calli from *D. floribunda*.

Embryogenic suspension cultures of *D. floribunda* were maintained in a medium with higher 2,4-D levels (4 mg/l) than used for Chinese yam (0.3–1 mg/l). In spite of the lower concentration of 2,4-D in the liquid proliferation medium for Chinese yam, embryogenic tissue was smaller and at a much earlier ontogenetic stage than *D. floribunda*. These embryogenic suspension cultures of Chinese yam have been maintained for more than 1 year, whereas embryogenic suspension cultures of *D. floribunda* could be maintained for only several subcultures.

The stimulation of germination by BA is not unique for Chinese yam. Gray and Mortensen [13] reported BA enhancement of germination of somatic embryos of grape. Exposure to low temperatures or gibberellic acid [14] or desiccation treatments [15] could also be used to break dormancy in grape. Ammirato [5] used 0.1 μ M zeatin for germination of *D. floribunda* somatic embryos. Additional effects of various amino acids [16] and abscisic acid [17] on somatic embryogenesis has been reported for many plant species. These treatments were either beneficial or essential for somatic embryo development or germination. Further modification of the culture medium used for Chinese yam somatic embryo development and germination could improve the efficiency of plant regeneration. To this point, over 50 plantlets have been regenerated from somatic embryos of Chinese yam.

Nodal stem section propagation described in this report has been considered to be the most feasible method for propagation of *Dioscorea* crops [11,12]. This rapidly-growing, regenerable embryogenic suspension culture could provide a much more efficient propaga-

tion system. Because embryogenic suspension culture tissue has been shown to be the best source of regenerable protoplasts in monocotyledonous species [18,19], and embryogenic suspension culture of yam may also be valuable for the development of a protoplast transformation and regeneration system. This embryogenic suspension culture may also be useful for both production of somaclonal variants and microprojectile-mediated transformation [20]. This rapidly-growing, maintainable and regenerable embryogenic suspension culture system may provide, for the first time, a vehicle for the genetic improvement of Chinese yam.

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